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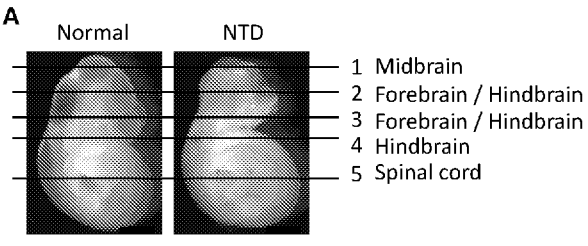
(54) Title: METHODS FOR PREVENTING NEURAL TUBE DEFECTS IN DIABETIC PREGNANCY

Fig. 1A



(57) Abstract: The invention provides compositions and methods for modulating levels of autophagy, as well as methods for preventing hyperglycemia-induced neural tube defects through administration of modulators of autophagy.

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TITLE OF THE INVENTION

Methods for Preventing Neural Tube Defects in Diabetic Pregnancy

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims priority to U.S. Provisional Application No. 62/501,398, filed May 4, 2017 which is hereby incorporated by reference herein in its entirety.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

This invention was made with government support under Grant Numbers DK101972 and DK103024 awarded by the National Institutes of Health. The government has certain rights in the invention.

BACKGROUND OF THE INVENTION

Incomplete neural tube closure results in neural tube defects (NTDs), severe birth defects of the central nervous system (Greene, N.D. et al., 2014, Annual review of Neuroscience, 37:221-242). Globally there are more than 300,000 NTD-affected pregnancies each year, and NTDs cause significant infant mortality and childhood morbidity. 1 out of 10 babies with NTDs will die before their first year. Annual medical and surgical costs for children born with NTDs in the US are more than $200 million. Preexisting maternal diabetes significantly increases the risk of NTDs (Greene, N.D. et al., 2014, Annual review of Neuroscience, 37:221-242; Correa, A. et al., 2008, American Journal of obstetrics and gynecology, 199:(237) e231-239; Becerra, J.E. et al., 1990, Pediatrics, 85:1-9; Ramos-Arroyo, M.A. et al., 1992, European Journal of Epidemiology, 8:503-508). Even under the best preconception care, diabetic women are five times more likely to have a child with birth defects than are nondiabetic women (Correa, A. et al., 2008, American Journal of obstetrics and gynecology 199:(237) e231-239). Therefore, there is an urgent need to develop effective interventions for this disease. Mechanistic studies are the first step in revealing potential therapeutic targets for diabetes-induced NTDs.

Autophagy, a catabolic cellular organelle process, removes unwanted cellular components through double-membrane autophagosomes fused with lysosomes, and, thus, is essential for survival, differentiation, development, and homeostasis (Codogno, P., 2014, Nature Reviews. Molecular Cell Biology, 15:153; Levine, B. et al., 2008, Cell, 132:27-42). Autophagy is required for embryonic neurulation because autophagy deficiency in Autophagy/beclin-1 regulator 1 (*AMBRA1*) null mutants results in massive neuroepithelial cell apoptosis and NTDs (Fimia, G.M. et al., 2007, Nature, 447:1121-1125), reminiscent of those observed in diabetic embryopathy. However, it is unclear how maternal diabetes represses autophagy during neurulation in the developing neuroepithelium. Protein kinases such as the mammalian target of rapamycin (mTOR) and the AMP-activated protein kinase (AMPK) are among the first discovered regulators for autophagy (Levine, B. et al., 2008, Cell, 132:27-42). Recent studies reported conflicting findings on the regulation of autophagy by the protein kinase C (PKC) signaling pathway. PKC activation is required for palmitic acid-induced autophagy *in vitro* (Tan, S.H. et al., 2012, The Journal of Biological Chemistry, 287:14364-14376). PKC inhibitors induce autophagy whereas PKC activators attenuate starvation- or rapamycin-induced autophagy *in vitro* (Jiang, H. et al., 2010, Biochemical and Biophysical Research Communication, 395: 471-476). The PKC family consists of twelve isoforms that control diverse physiological and pathophysiological functions, including cell proliferation, differentiation and apoptosis (Mochly-Rosen, D. et al., 2012, Nature Reviews. Drug Discovery, 11:937-957). Maternal diabetes-induced neuroepithelial cell apoptosis is the central mechanism underlying diabetes-induced NTDs (Yang et al., 2013, Sci Signal. 6(290):ra74; Xu, C. et al., 2013, American Journal of Physiology 305: E667-678; Li, X. et al., 2013, Diabetes, 62:599-608; Wu, Y. et al., 2015, Diabetes, 64:2526-2536). Definitive molecular evidence supporting the key role of specific PKC isoforms in diabetic embryopathy is lacking, and the molecular intermediates downstream of PKC have not been characterized.

The PPAR-γ coactivator 1α (PGC-1α) regulates mitochondrial function and cell viability (Wu, Z. et al., 1999, Cell, 98:115-124; Luo, Y. et al., 2009, Journal of Molecular Neuroscience: MN, 39: 262-268; Adhihetty, P. J. et al., 2009, American Journal of Physiology. Cell Physiology, 297: C217-225). PGC-1α is abundantly present

in the central nervous system (Luo, Y. et al., 2009, Journal of Molecular Neuroscience: MN, 39: 262-268). Furthermore, overexpression of PGC-1α suppresses apoptosis (Luo, Y. et al., 2009, Journal of Molecular Neuroscience: MN, 39: 262-268; Adhihetty, P. J. et al., 2009, American Journal of Physiology. Cell Physiology, 297: C217-225), whereas reduced levels of PGC-1α sensitize cells to apoptosis (Liang, J. et al., 2010, Journal of Neuroscience Research, 88: 1918-1925). Mitochondrial dysfunction and apoptosis are interdependent and causative events in diabetic embryopathy (Yang et al., 2013, Sci Signal. 6(290):ra74; Xu, C. et al., 2013, American Journal of Physiology 305: E667-678).

Thus, there is a need in the art for development of effective interventions for NTDs. The present invention addresses this need.

SUMMARY OF THE INVENTION

In one embodiment, the invention relates to a method for treating or preventing a hyperglycemia-induced neural tube defect or a disease or disorder associated with hyperglycemia-induced neural tube defects, comprising administering to a subject a composition comprising an agonist of autophagy.

In one embodiment, the agonist of autophagy is an activator of PGC-1α, an activator of PPAR-γ, or an activator of Sirtuin-1.

In one embodiment, the activator is a protein, a peptide, a peptidomimetic, an antibody, a ribozyme, an organic compound, an inorganic compound, a small molecule, a nucleic acid, a vector, or an antisense nucleic acid molecule.

In one embodiment, the activator of PGC-1α is ZLN005, telmisartan or fenofibrate.

In one embodiment, the activator of PPAR-γ is thiazolidinedione, rosiglitazone, pioglitazone, honokiol, amorfrutin 1, amorfrutin B, amorphastilbol, roscovitine, aleglitazar, muraglitazar, saroglitazar or tesaglitazar.

In one embodiment, the activator of Sirtuin-1 is Resveratrol, resVida, Lonevinex, SRT501, Pterostilbene, SRT1720, SRT2104, SRT2379, berberine, acetylsalicylic acid, Metformin, AICAR, AZD-769662 oxaloacetate, or rapamycin.

In one embodiment, the agonist of autophagy is 5,6- epoxyeicosatrienoic acid (EET), 8,9-EET, 11,12-EET, 14,15-EET, epoxyeicosatrienoic acid analogue EET-A, or (±)14(15)-EET.

In one embodiment, the agonist of autophagy is an inhibitor of PKCα. In one embodiment, the inhibitor is a protein, a peptide, a peptidomimetic, an antibody, a ribozyme, an organic compound, an inorganic compound, a small molecule, a nucleic acid, a vector, or an antisense nucleic acid molecule.

In one embodiment, the inhibitor of PKCα is Go6976, Bryostatin 1, Enzastaurin, Staurosporine, Bisindolylmaleimide I, Ro 31-8220 mesylate, Ro 32-0432 hydrochloride, Sotrastaurin, N,N-Dimethyl-D-erythro-sphingosine, PKC412, H 9 dihydrochloride, 10Z-Hymenialdisine, ML-9, HA-156, Bisindolylmaleimide XI hydrochloride, (±)-Palmitoylcarnitine chloride, HBDDE, GF 109203X hydrochloride, HA 100 dihydrochloride, Hypericin, Bisindolylmaleimide X hydrochloride, Bisindolylmaleimide II, Bisindolylmaleimide III, Bisindolylmaleimide IV, NPC-15437, (2S,3R,4E)-2-Azido-3-(tert-butyldimethylsilyl)-erythro-sphingosine, 1-(5-Isoquinolinesulfonyl)-3-methylpiperazine hydrochloride, 1-(5-Isoquinolinesulfonyl)piperazine hydrochloride, 1,2,3,4-Tetrahydrostaurosporine, α-Acetamidocinnamic acid, Bisindolylmaleimide VIII acetate, Cercosporin, Daphnetin, Dequalinium chloride, Hypocrellin A, N-(5-Amino-2-methylphenyl)-4-(3-pyridyl)-2-pyrimidineamine, TMB-8, Verbascoside, Calphostin C, D-erythro-Dihydrosphingosine, PLA2 and PLD inhibitor, D,L-erythro-Dihydrosphingosine, sphingosine kinase inhibitor, L-threo-Dihydrosphingosine or Rottlerin.

In one embodiment, the agonist of autophagy is an inhibitor of miR-129-2. In one embodiment, the inhibitor is a small molecule, an anti-miR oligonucleotide (AMO), and a miR sponge.

In one embodiment, the subject has been diagnosed as having a condition associated with hyperglycemia. In one embodiment, the subject is diabetic.

In one embodiment, the subject is pregnant or trying to conceive.

In one embodiment, the method prevents or reduces the occurrence of a hyperglycemica-induced neural tube defect in an embryo or fetus.

In one embodiment, the disease or disorder associated with hyperglycemia-induced neural tube defects is congenital heart defect, congenital heart disease, spina bifida, exencephaly, craniorachischisis, microcephaly, chiari malformation, anencephaly, fetal or infant death or a combination thereof.

BRIEF DESCRIPTION OF THE DRAWINGS

The following detailed description of embodiments of the invention will be better understood when read in conjunction with the appended drawings. It should be understood that the invention is not limited to the precise arrangements and instrumentalities of the embodiments shown in the drawings.

Figure 1, comprising Figure 1A through Figure 1C, depicts the results of example experiments demonstrating neuroepithelial cell apoptosis in normal and NTD embryos. Figure 1A depicts indicators of histological sectioning plane for Figure 1B. E10.5 embryos were used for apoptosis detection. Red lines indicate the levels of sectioning shown on the right. For each embryo, five sections from top to bottom were chosen to detect apoptotic cells in the forebrain, midbrain, hindbrain and spinal cord were indicated in each picture. Figure 1B depicts, representative TUNEL assay images showing apoptotic cells (red dots) in sections of the forebrain, midbrain, hindbrain and spinal cord. Cell nuclei were stained with DAPI (blue). Scale bars: 300 μm, and 70 μm for the enlarged boxed areas. Figure 1C depicts, quantification of TUNEL positive cells per section (three serial sections of each area per embryo, and three embryos from three dams were analyzed) in the corresponding brain regions. ND: nondiabetic dams; DM: diabetic mellitus dams; NTD: exencephaly. * indicate significant difference ($P < 0.05$) compared with the ND groups.

Figure 2, comprising Figure 2A through Figure 2C, depicts the results of example experiments demonstrating autophagy in the developing neuroepithelia of normal and NTD embryos. Figure 2A depicts indicators of histological sectioning plane for Figure 2B. Red lines indicate the levels of sections shown below. For each embryo, five sections from top to bottom were chosen to detect autophagy levels, forebrain, midbrain, hindbrain and spinal cord were indicated in each picture. Figure 2B depicts, representative images showing autophagy levels (green LC3-GFP puncta) in sections of

the forebrain, midbrain, hindbrain and spinal cord. E10.5 embryos from nondiabetic (ND) and diabetic mellitus (DM) females mated with GFP-LC3 transgenic males were used for autophagy examination. Scale bar: 300 μm. Individual green LC3-GFP puncta can be clearly visualized in high magnification images in Figure 4. Figure 2C depicts, quantification of relative autophagy levels by fluorescence intensity using the Image J software in midbrain and forebrain (three serial sections of each area per embryo, and three embryos from three dams were analyzed) in the corresponding brain regions. ND: nondiabetic embryos; DM: diabetic mellitus embryos; NTD: exencephaly. * indicate significant difference ($P < 0.05$) compared with the ND groups.

Figure 3, comprising Figure 3A through Figure 3D, depicts the results of example experiments demonstrating SOD1 blocks diabetes-induced ROS and *Prkca* deletion ameliorates diabetes-induced NTDs. Figure 3A depicts, Dihydroethidium (DHE) staining and quantification for superoxide (Figure 3A) and immunostaining of phosphorylated PKCα (Figure 3B) in the E8.75 neuroepithelium (the V shape structure) of embryonic sections. All cell nuclei were stained with DAPI (blue). Bars = 70 μm. In Figure 3A and Figure 3B, three embryos from three different dams each group were analyzed and quantified by fluorescence intensity using the Image J software (N = 3). Figure 3C depicts morphology of E10.5 embryos from ND WT, ND *Prkca*$^{-/-}$, DM WT and DM *Prkca*$^{-/-}$ mice, and NTD rates in E10.5 embryos. Scale bars = 1 mm. The red arrow indicates exencephaly. Lower panel: frontal sections of embryos in the upper panel showing an open neural tube. Figure 3D depicts, blood glucose concentrations. N for (Figure 3C and Figure 3D) the numbers (N) of embryos per condition and statistical analyses are indicated in Figure 16. WT: wild-type; ND: nondiabetic; DM: diabetic mellitus. * indicate significant difference ($P < 0.05$) compared with the ND groups.

Figure 4, comprising Figure 4A through Figure 4F, depicts the results of example experiments demonstrating *Prkca* deletion reverses maternal diabetes-induced autophagy impairment. Figure 4A depicts two autophagosomes in E8.75 neuroepithelial cells in EM micrographs. Bar = 100 μm. EM pictures were taken with an electron microscope (model: Joel JEM-1200EX) under 12K resolution. Each image covered 9.73 um^2 areas. The numbers of autophagosome and cell on each image were counted to get the value of autophagosome per cell. Five images of each embryo and three embryos

from different mothers were quantified for each group. Total eighty-six autophagosomes from the four groups were counted. Figure 4B depicts quantitative data of GFP-LC3 punctate foci (green dots) with a diameter greater or equal to 20 pixels in E8.75 neuroepithelial cells of embryonic sections. Cell nuclei were stained with DAPI (blue). Bars = 3.5 μM. Figure 4C depicts, LC3-II abundance in E8.75 embryos. All experiments were performed using three embryos from three different dams (N = 3) and graphs in Figure 4A, Figure 4B and Figure 4C showed summaries of all data. Figure 4D, Figure 4E, and Figure 4F depicts representative images and quantification of autophagosomes in C17.2 neural stem cells using Cyto-ID staining (Green). caPKCα: constitutively active PKCα. pcDNA3: backbone vector for caPKCα. SAG (1-Stearoyl-2-arachidonoyl-sn-glycerol): a pharmacological PKCα activator. DMSO: a vehicle control for SAG. Control: 5 mM glucose; High glucose (HG): 25 mM glucose. In Figure 4F, PKCα siRNA knockdown reversed high glucose-suppressed autophagy. In Figure 4D, Figure 4E, and Figure 4F experiments were repeated three times (N = 3). Bars = 15 μM. *indicate significant difference compared with other group or groups.

Figure 5, comprising Figure 5A through 5E, depicts the results of example experiments demonstrating that deletion of the *Prkca* gene restores mitochondrial function. Figure 5A depicts, defective mitochondria (Mito) rates and total number of mitochondria in embryonic sections. Defective mitochondria rate = number of defective mitochondria divided by total number of mitochondria per image area (image size: 9.43 μm^2) in neuroepithelial cells based on the images of defective and normal mitochondria in Figure 11. Neuroepithelia from three embryos (N = 3) derived from different dams were used. Three serial sections per embryo were analyzed. The effect of *Prkca* gene deletion on protein abundance of Bak (Figure 5B), Bax (Figure 5C), Puma (Figure 5D), and Bim (Figure 5E) in isolated mitochondria of E8.75 embryos. Bar graphs for protein abundance were quantitative data from three independent experiments. * indicate significant difference compared with other groups.

Figure 6, comprising Figure 6A through Figure 6E, depicts the results of example experiments demonstrating that *Prkca* gene deletion restores cellular homeostasis and prevents apoptosis. Protein abundance of phosphorylated (p-) PERK and p-IRE1α (Figure 6A); p-eIF2α and CHOP (Figure 6B); cleaved caspase 8 (Figure 6C) and caspase

3 (Figure 6D) in E8.75 embryos. Bar graphs for protein abundance were quantitative data from three independent experiments. Figure 6E depicts, representative images of the TUNEL assays in E8.75 embryos. Apoptotic cells were labeled in red and nuclei were labeled in blue by DAPI. The dense blue V shape areas are the neural plate. The level of the body axis was at hindbrain level. The bar graph showed the quantification of apoptotic cell number. Experiments were repeated using 5 embryos (N = 5) from different dams and five images were obtained from each embryo. Scale bars = 70 μm. WT: wild-type; ND: nondiabetic; DM: diabetic mellitus. * indicate significant difference compared with other groups.

Figure 7, comprising Figure 7A through Figure 7M, depicts the results of example experiments demonstrating PKCα mediates the inhibitory effect of diabetes on PGC-1α through miR-129-2 up-regulation. The effect of *Prkca* deletion ($PKC\alpha^{-/-}$) on *PPARGC1A* (PGC-1α) mRNA (Figure 7A) and PGC-1α protein abundance (Figure 7B) in E8.75 embryos (N = 3) from different dams. WT: wild-type; ND: nondiabetic; DM: diabetic mellitus. PGC-1α protein abundance in neural stem cell cultures influenced by PKCα siRNA knockdown (Figure 7C) and constitutively active PKCα (caPKCα) (Figure 7D). PGC-1α protein and mRNA abundance affected by the miR-129-2 mimic (Figure 7E, Figure 7F) and the miR-129-2 inhibitor (Figure 7G, Figure 7H) in C17.2 neural stem cells. (Figure 7I, Figure 7J) Levels of miR-129-2 and PGC-1α mRNA in an RNA immunoprecipitation assay. The RNA-induced silencing complex (RISC) in E8.75 embryos was pulled down by the AGO2 antibody, and then the levels of miR-129-2 and PGC-1α mRNA was detected in input and immunoprecipitates. caPKCα mimicked the stimulatory effect of high glucose (25 mM) on miR-129-2 expression (Figure 7K). PKCα siRNA knockdown blocked high glucose-induced miR-129-2 expression (Figure 7L). In Figure 7C, Figure 7D, Figure 7E, Figure 7F, Figure 7G, Figure 7H, Figure 7I, Figure 7J, Figure 7K and Figure 7L, experiments were preformed independently three times (N = 3). The effect of *Prkca* deletion on miR-129-2 abundance (Figure 7M) using three embryos (N = 3) from different dams. In Figure 7I and Figure 7J, three litters (N = 3) of each group were used. * indicate significant difference compared with other groups.

Figure 8, comprising Figure 8A through Figure 8H, depicts the results of example experiments demonstrating that PPARGC1A overexpression restores autophagy

and suppresses NTD formation. Figure 8A depicts, the transgene construct for the *PPARGC1A* (PGC-1α) transgenic mouse line, and green signals of GFP protein in the V shape neural plate of an E8.5 *PPARGC1A*$^{+}$ embryo. The level of the body axis was at hindbrain level. Figure 8B depicts, autophagosome numbers in E8.75 neuroepithelial cells of embryonic sections. Five images of each embryo and three embryos from different mothers were quantified for each group. Total eighty-one autophagosomes from the four groups were counted. Figure 8C depicts LC3-II abundance in E8.75 embryos. Figure 8D depicts qquantitative data of GFP-LC3 punctate foci (green dots) in E8.75 neuroepithelial cells. Cell nuclei were stained with DAPI (blue). Bars = 3.5 μM. All experiments were performed independently three times (N = 3) using embryos from different dams, and graphs in Figure 8B, Figure 8C, and Figure 8D showed summaries of all data. Figure 8E depicts representative images and quantitative data of GFP-LC3 punctate foci (green dots) with a diameter greater or equal to 20 pixels, and mitochondrial mass (Mito-ID red signals) in GFP-LC3 HeLa reporter cells. Bars = 15 μm. * indicate significant difference compared to the previous time point,. Figure 8F depicts representative images of Cyto-ID staining puncta, which represented autophagosomes, and quantification of puncta. Bars = 3.5 μM. In Figure 8E and Figure 8F, experiments were performed three times (N = 3). Figure 8G depicts, blood glucose concentrations from nondiabetic (ND) and diabetic mellitus (DM) mated with PGC-1α transgenic males. Figure 8H depicts, NTD rates in E10.5 embryos. N for Figure 8G and Figure 8H was indicated in Figure 17.

Figure 9, comprising Figure 9A through Figure 9G, depicts the results of example experiments that PPARGC1A overexpression prevents diabetes-induced cellular organelle stress and apoptosis. Figure 9A depicts, total number of mitochondria (Mt) and percentages of defective mitochondria (number of defective mt divided by total number of mt) from wild-type (WT) and PGC-1α overexpressing embryos. PGC-1α transgenic males mated with nondiabetic (ND) and diabetic (DM) females to generate WT and PGC-1α overexpressing embryos. Figure 9B depicts, mRNA abundance of mitochondrial genes in whole embryos: Cox5b, Nrf1, Tfam, Sox2. Protein abundance of p-IRE1α and CHOP (Figure 9C), cleaved caspase 8 (Figure 9D) and caspase 3 (Figure 9E) in E8.75 embryos. Bar graphs for protein abundance were quantitative data from three independent

experiments. Representative images of the TUNEL assays in E8.75 embryos (Figure 9F). Apoptotic cells were labeled in red and nuclei were labeled by DAPI in blue. The dense blue V shape areas are the neural plates. The level of the body axis was at hindbrain level. The bar graph showed the quantification of apoptotic cell number. Experiments were repeated using 5 embryos (N = 5) from different dams and five images were obtained from each embryo. Scale bars are 30 μm. In Figure 9A, Figure 9B, Figure 9C, Figure 9D and Figure 9E, experiments were repeated three times using embryos from three different dams (N = 3) per group. * indicate significant differences ($P < 0.05$) compared to the other groups. Figure 9G is a schematic diagram depicting the maternal diabetes-induced pathway, PKCα-miR-129-2-PGC-1α, in autophagy impairment leading to mitochondrial dysfunction, ER stress, apoptosis and NTD formation. PKCα up-regulates miR-129-2, which in turn down-regulates PGC-1α. PKCα and miR-129-2 suppress autophagy whereas PGC-1α stimulates autophagy.

Figure 10, comprising Figure 10A through Figure 10C, depicts the results of example experiments demonstrating characteristics of NTDs on the mouse model of diabetic embryopathy. Figure 10A depicts, types of NTDs in E10.5 embryos exposed to maternal diabetes. Figure 10B depicts, indicators of histological sectioning plane for Figure 10C. Red lines indicate the levels of sectioning shown below. For each embryo, five sections from top to bottom were chosen to show the morphologic characteristics of the forebrain, midbrain, hindbrain and spinal cord. Figure 10C depicts, HE staining images of the forebrain, midbrain, hindbrain and spinal cord structures. In Figure 10B and Figure 10C, NTD means exencephaly. Scale bar: 300 μm. ND: nondiabetic dams; DM: diabetic mellitus dams; NTD: neural tube defects.

Figure 11, comprising Figure 11A through Figure 11F, depicts the results of example experiments demonstrating *Prkca* gene deletion maternal diabetes-induced autophagy gene alteration and mitochondrial dysfunction. Figure 11A depicts mRNA abundance of ULK1, ATG5, BECN1, p62 and Bnip3. Figure 11B and Figure 11C depicts, the abundance of PKCα protein (Figure 11B) and mRNA (Figure 11C) after cells were transfected with control (ctrl) siRNA or PKCα siRNA at different concentrations. 25 nM PKCα siRNA reduced about 65% endogenous PKCα protein expression and this concentration was chosen for subsequent experiments. Morphology of neuroepithelial

cell mitochondria of E8.75 embryos from nondiabetic wild-type (ND-WT), ND-*Prkca*$^{-/-}$, diabetic wild-type (DM-WT) and DM-*Prkca*$^{-/-}$ dams. Figure 11D depicts, normal mitochondria having transversely oriented cristae enclosed by intact outer membranes. Defective mitochondria with disarrayed or disruptive cristae and decreased electronic density of the matrix in the DM-WT group. Scale bar: 200 nm. Figure 11E and Figure 11F depicts, protein abundance of phospo (p)-Bad (Figure 11E) and tBid (Figure 11F). Experiments were repeated three times using three E8.75 embryos from nondiabetic wild-type (ND-WT), ND-*Prkca*$^{-/-}$, diabetic wild-type(DM-WT) and DM-*Prkca*$^{-/-}$ dams (n = 3) per group. * mean significant difference ($P < 0.05$) compared to other groups.

Figure 12, comprising Figure 12A through Figure 12B, depicts the results of experimental example demonstrating *Prkca* gene deletion reverses diabetes-increased ER chaperone gene expression and XBP1 splicing event. Figure 12A depicts, mRNA abundance of six ER chaperone genes: BiP, Calnexin, CHOP, PDIA, GRP94, IRE1α. Experiments were repeated three times using embryos from three different dams (n = 3) per group. * indicates significant differences ($P < 0.05$) compared to the other groups. Figure 12B depicts XBP1 splicing in E8.75 embryos from nondiabetic wild-type (ND-WT), ND-*Prkca*$^{-/-}$, diabetic wild-type(DM-WT) and DM-*Prkca*$^{-/-}$ dams.

Figure 13, comprising Figure 13A through Figure 13G, depicts the results of example experiments demonstrating miR-129-2 binds to the 3'UTR of PGC-1α mRNA and represses PGC-1α expression. Figure 13A depicts, Schematic representation of the PGC-1α mRNA depicting miR-129-2-3p binding sites in its 3'-UTR. One predicted miR-129-2-3p binding site (position 3052-3072) is located in the 3'-UTR of PGC-1α mRNA. The abundance of PGC-1α mRNA after 48 hours following biotin-miR-129-2 transfection was shown. Figure 13B depicts, schematic of plasmids of different chimeric firefly luciferase PGC-1α reporters. Relative luciferase reporter activities driven by the CR (coding region), 3'UTR and BS (a 3'-UTR fragment encompassing the specific miR-129-2 binding site (BS) or having the BS deleted (Mut)) after ectopic overexpression of miR-129-2-3p were shown in the bar graph. Luciferase reporter activities were normalized to the Renilla luciferase activities. Values were the means ± SE from three separate experiments. * indicate significant differences ($P < 0.05$) compared with the scramble group. Figure 13C and Figure 13D depicts, miR129-2-3p abundance (Figure

13C) and PGC1α protein abundance (Figure 13D) in C17.2 neural stem cells transfected with the control (ctrl) mimic or the miR-129-2 mimic. Figure 13E and Figure 13F depicts, miR129-2-3p abundance (Figure 13E) and PGC1α protein abundance (Figure 13F) in C17.2 neural stem cells transfected with the control (ctrl) inhibitor or the miR-129-2 inhibitor. Figure 13G depicts, miR-129-2 levels in C17.2 neural stem cells cultured for 48 hours under normal glucose (5 mM glucose) or high glucose (14, 20, 25 and 33 mM glucose) conditions. High mannitol (9, 15, 20 and 28 mM mannitol) along with 5 mM glucose served osmotic controls of high glucose. Experiments were repeated three times (n = 3) and the quantification of the data were shown in the bar graph. * indicate significant differences ($P < 0.05$) compared with the control groups.

Figure 14, comprising Figure 14A through Figure 14E, depicts results of example experiments demonstrating in vitro PGC-1α overexpression induces autophagosome and restores autophagy suppressed by high glucose. Figure 14A depicts, PGC-1α gene overexpression restores the expression levels of maternal diabetes-suppressed autophagy-related gene: mRNA abundance of ULK1, ATG5, BECN1, p62, and Bnip3 in wild-type (WT) and PGC-1α overexpression (PGC-1α^{+}) embryos. PGC-1α transgenic males mated with nondiabetic (ND) and diabetic (DM) females to generate WT and PGC-1α overexpressing embryos. Experiments were repeated three times using different embryos from three dams (n = 3) per group. * mean significant differences ($P < 0.05$) compared to other groups. Figure 14B depicts that autophagosome (GFP punctate) formation was stimulated by PGC-1α tranfections (anti-Flag staining-Red). pcDNA3 blank vector transfections served as controls. Nuclei were counterstained by DAPI. Scale bars:15 μm. The bar graph showed quantification of GFP punctate. Figure 14C and Figure 14D depicts that PGC-1α siRNA effectively silences PGC-1α. PGC-1α protein abundance (Figure 14C) and mRNA abundance (Figure 14D) in cells transfected with the control (ctrl) siRNA or the PGC-1α siRNA at different concentrations. Experiments were repeated three times (n = 3) and quantification of the data were shown in the bar graph. * indicate significant differences ($P < 0.05$) compared with the control group. Figure 14E depicts representative images of Cyto-ID staining puncta, which represented autophagosomes. PGC-1α staining (anti-Flag staining-Red) showed PGC-1α overexpression upon PGC-1α vector transfections. Top row 1 and 2 showed pcDNA3

blank vector transfections as controls. Nuclei were counterstained by DAPI. Scale Bars: 15 μm. The bar graph showed quantification of Cyto-ID staining puncta that represents autophagosome. Five cells per group were quantified and experiments were repeated three times. * indicate significant difference compared with other three groups and # depict significant difference compared with the two 5 mM glucose groups.

Figure 15, comprising Figure 15A through Figure 15C, depicts results of example experiments demonstrating that PGC-1α gene overexpression suppresses maternal diabetes-induced ER stress. Figure 15A depicts protein abundance of p-PERK and p-eIF2α. Experiments were repeated three times using embryos from three different dams (n = 3) per group. Figure 15B depicts, XBP1 mRNA splicing was detected in E8.75 embryos by reverse transcription and subsequent PCR. n = 2 means two embryos from separate dams per group. Figure 15C depicts mRNA abundance of ER chaperone genes: Calnexin, BiP, CHOP, PDIA, GPR94 and IRE1α. Experiments were repeated three times using embryos from three different dams (n = 3) per group. * indicate significant differences ($P < 0.05$) compared to the other three groups.

Figure 16 depicts results of example experiments demonstrating that targeted gene deletion of *Prkca* ameliorates diabetes-induced neural tube defects (NTDs). * indicates significant difference when compared to other groups by using *Chi*-square test. (ND: nondiabetic; DM: diabetic; WT: wild-type; $^{-/-}$: knockout; ♂: male; ♀: female)

Figure 17 depicts results of example experiments demonstrating that PGC-1α overexpression ameliorates diabetes-induced neural tube defects. * indicates significant difference when compared to other groups by using *Chi*-square test. (ND: nondiabetic; DM: diabetic; WT: wild-type; $^{-/-}$: knockout; ♂: male; ♀: female)

DETAILED DESCRIPTION

The invention is based in part on the discovery that autophagy regulators PKCα and miR-129-2 mediate the teratogenicity of hyperglycemia that can lead to NTDs. Further, the invention is based in part on the discovery that PKCα increases the expression of miR-129-2, which represses autophagy by directly targeting PCG-1α, a positive regulator for mitochondrial function that is disturbed by maternal diabetes. PCG-1α supports neurulation by stimulating autophagy in neuroepithelial cells.

Therefore in various embodiments, the invention provides compositions and methods for treating or preventing hyperglycemia-associated neural tube defects through modulating at least one autophagy regulator, at least one regulator of mitochondrial function, or the interaction of at least one at least one autophagy regulator with at least one regulator of mitochondrial function.

In one embodiment, the invention provides methods of treating or preventing hyperglycemia-induced neural tube defects, or a disease or disorder associated with hyperglycemia-induced neural tube defects in a subject or in an offspring of a subject through administration of the modulator of the invention to the subject. A range of diseases including, but not limited to, congenital heart defects, congenital heart disease, spina bifida, exencephaly, craniorachischisis, microcephaly, chiari malformation, anencephaly and fetal or infant death are associated with hyperglycemia-induced neural tube defects.

Definitions

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs.

As used herein, each of the following terms has the meaning associated with it in this section.

The articles "a" and "an" are used herein to refer to one or to more than one (*i.e.*, to at least one) of the grammatical object of the article. By way of example, "an element" means one element or more than one element.

"About" as used herein when referring to a measurable value such as an amount, a temporal duration, and the like, is meant to encompass variations of ±20%, ±10%, ±5%, ±1%, or ±0.1% from the specified value, as such variations are appropriate to perform the disclosed methods.

The term "abnormal" when used in the context of organisms, tissues, cells or components thereof, refers to those organisms, tissues, cells or components thereof that differ in at least one observable or detectable characteristic (e.g., age, treatment, time of day, etc.) from those organisms, tissues, cells or components thereof that display the

"normal" (expected) respective characteristic. Characteristics which are normal or expected for one cell or tissue type, might be abnormal for a different cell or tissue type.

The term "activate," as used herein, means to induce or increase an activity or function relative to a control value. For example, the activity is induced or increased by at least about 5%, 10%, 25%, 50%, 75%, 95% or by more than 95% compared to a control. "Activate," as used herein, also means to increase a molecule, a reaction, an interaction, a gene, an mRNA, and/or a protein's expression, stability, function or activity by a measurable amount or to increase entirely. Activators are compounds that, e.g., bind to, partially or totally induce stimulation, increase, promote, induce activation, activate, sensitize, or up regulate a protein, a gene, and an mRNA stability, expression, function and activity, e.g., agonists.

The term "activity" of a compound of the invention includes all activities elicited by compound of the inventions in a responsive cell. It includes genomic and non-genomic activities elicited by these compounds. In certain embodiments, biological activities refers to phenotypic changes. In certain embodiments, biological activity refers to cytotoxicity, inhibition of autophagy, stimulation of autophagy, inhibition of mitochondrial activity, or stimulation of mitochondrial activity.

"Antisense" refers particularly to the nucleic acid sequence of the non-coding strand of a double stranded DNA molecule encoding a protein, or to a sequence which is substantially homologous to the non-coding strand. As defined herein, an antisense sequence is complementary to the sequence of a double stranded DNA molecule encoding a protein. It is not necessary that the antisense sequence be complementary solely to the coding portion of the coding strand of the DNA molecule. The antisense sequence may be complementary to regulatory sequences specified on the coding strand of a DNA molecule encoding a protein, which regulatory sequences control expression of the coding sequences.

A "disease" is a state of health of an animal wherein the animal cannot maintain homeostasis, and wherein if the disease is not ameliorated then the animal's health continues to deteriorate.

In contrast, a "disorder" in an animal is a state of health in which the animal is able to maintain homeostasis, but in which the animal's state of health is less

favorable than it would be in the absence of the disorder. Left untreated, a disorder does not necessarily cause a further decrease in the animal's state of health.

A disease or disorder is "alleviated" if the severity of a sign or symptom of the disease or disorder, the frequency with which such a sign or symptom is experienced by a patient, or both, is reduced.

An "effective amount" or "therapeutically effective amount" of a compound is that amount of a compound which is sufficient to provide a beneficial effect to the subject to which the compound is administered. An "effective amount" of a delivery vehicle is that amount sufficient to effectively bind or deliver a compound.

The term "inhibit," as used herein, means to suppress or block an activity or function, for example, about ten percent relative to a control value. For example, the activity is reduced or decreased by at least about 5%, 10%, 25%, 50%, 75%, 95% or by more than 95% compared to a control. "Inhibit," as used herein, also means to reduce a molecule, a reaction, an interaction, a gene, an mRNA, and/or a protein's expression, stability, function or activity by a measurable amount or to prevent entirely. Inhibitors are compounds that, e.g., bind to, partially or totally block stimulation, decrease, prevent, delay activation, inactivate, desensitize, or down regulate a protein, a gene, and an mRNA stability, expression, function and activity, e.g., antagonists.

As used herein, an "instructional material" includes a publication, a recording, a diagram, or any other medium of expression which can be used to communicate the usefulness of a compound, composition, vector, or delivery system of the invention in the kit for effecting alleviation of the various diseases or disorders recited herein. Optionally, or alternately, the instructional material can describe one or more methods of alleviating the diseases or disorders in a cell or a tissue of a mammal. The instructional material of the kit of the invention can, for example, be affixed to a container which contains the identified compound, composition, vector, or delivery system of the invention or be shipped together with a container which contains the identified compound, composition, vector, or delivery system. Alternatively, the instructional material can be shipped separately from the container with the intention that the instructional material and the compound be used cooperatively by the recipient.

By the term "modulating," as used herein, is meant mediating a detectable increase or decrease in the level of a response in a subject compared with the level of a response in the subject in the absence of a treatment or compound, and/or compared with the level of a response in an otherwise identical but untreated subject. The term encompasses perturbing and/or affecting a native signal or response thereby mediating a beneficial therapeutic response in a subject, preferably, a human. The term includes increases or decreases in an activity of a cell (e.g., cellular element degradation by autophagy or mitochondrial activity) in response to exposure to a compound described herein, e.g., the inhibition of autophagy in at least a sub-population of cells in a subject such that a desired end result is achieved, e.g., a therapeutic result.

As used herein, a "modulator" is a compound that modifies the expression, activity or biological function of a target molecule as compared to the expression, activity or biological function of the target molecule in the absence of the modulator.

The terms "patient," "subject," "individual," and the like are used interchangeably herein, and refer to any animal, or cells thereof whether in vitro or in vivo, amenable to the methods described herein. In certain non-limiting embodiments, the patient, subject or individual is a human.

A "therapeutic" treatment is a treatment administered to a subject who exhibits signs or symptoms of a disease or disorder, for the purpose of diminishing or eliminating those signs or symptoms.

As used herein, "treating a disease or disorder" means reducing the severity and/or frequency with which a sign or symptom of the disease or disorder is experienced by a patient.

The phrase "biological sample" as used herein, is intended to include any sample comprising a cell, a tissue, or a bodily fluid in which expression of a nucleic acid or polypeptide is present or can be detected. Samples that are liquid in nature are referred to herein as "bodily fluids." Biological samples may be obtained from a patient by a variety of techniques including, for example, by scraping or swabbing an area of the subject or by using a needle to obtain bodily fluids. Methods for collecting various body samples are well known in the art.

As used herein, an "immunoassay" refers to any binding assay that uses an antibody capable of binding specifically to a target molecule to detect and quantify the target molecule.

By the term "specifically binds," as used herein with respect to an antibody, is meant an antibody which recognizes a specific antigen, but does not substantially recognize or bind other molecules in a sample. For example, an antibody that specifically binds to an antigen from one species may also bind to that antigen from one or more species. But, such cross-species reactivity does not itself alter the classification of an antibody as specific. In another example, an antibody that specifically binds to an antigen may also bind to different allelic forms of the antigen. However, such cross reactivity does not itself alter the classification of an antibody as specific.

In some instances, the terms "specific binding" or "specifically binding," can be used in reference to the interaction of an antibody, a protein, or a peptide with a second chemical species, to mean that the interaction is dependent upon the presence of a particular structure (e.g., an antigenic determinant or epitope) on the chemical species; for example, an antibody recognizes and binds to a specific protein structure rather than to proteins generally. If an antibody is specific for epitope "A", the presence of a molecule containing epitope A (or free, unlabeled A), in a reaction containing labeled "A" and the antibody, will reduce the amount of labeled A bound to the antibody.

A "coding region" of a gene consists of the nucleotide residues of the coding strand of the gene and the nucleotides of the non-coding strand of the gene which are homologous with or complementary to, respectively, the coding region of an mRNA molecule which is produced by transcription of the gene.

A "coding region" of a mRNA molecule consists of the nucleotide residues of the mRNA molecule which are matched with an anti-codon region of a transfer RNA molecule during translation of the mRNA molecule or which encode a stop codon. The coding region may thus include nucleotide residues comprising codons for amino acid residues which are not present in the mature protein encoded by the mRNA molecule (e.g., amino acid residues in a protein export signal sequence).

"Complementary" as used herein to refer to a nucleic acid, refers to the broad concept of sequence complementarity between regions of two nucleic acid strands

or between two regions of the same nucleic acid strand. It is known that an adenine residue of a first nucleic acid region is capable of forming specific hydrogen bonds ("base pairing") with a residue of a second nucleic acid region which is antiparallel to the first region if the residue is thymine or uracil. Similarly, it is known that a cytosine residue of a first nucleic acid strand is capable of base pairing with a residue of a second nucleic acid strand which is antiparallel to the first strand if the residue is guanine. A first region of a nucleic acid is complementary to a second region of the same or a different nucleic acid if, when the two regions are arranged in an antiparallel fashion, at least one nucleotide residue of the first region is capable of base pairing with a residue of the second region. Preferably, the first region comprises a first portion and the second region comprises a second portion, whereby, when the first and second portions are arranged in an antiparallel fashion, at least about 50%, and preferably at least about 75%, at least about 90%, or at least about 95% of the nucleotide residues of the first portion are capable of base pairing with nucleotide residues in the second portion. More preferably, all nucleotide residues of the first portion are capable of base pairing with nucleotide residues in the second portion.

"Isolated" means altered or removed from the natural state. For example, a nucleic acid or a peptide naturally present in its normal context in a living animal is not "isolated," but the same nucleic acid or peptide partially or completely separated from the coexisting materials of its natural context is "isolated." An isolated nucleic acid or protein can exist in substantially purified form, or can exist in a non-native environment such as, for example, a host cell.

An "isolated nucleic acid" refers to a nucleic acid segment or fragment which has been separated from sequences which flank it in a naturally occurring state, i.e., a DNA fragment which has been removed from the sequences which are normally adjacent to the fragment, i.e., the sequences adjacent to the fragment in a genome in which it naturally occurs. The term also applies to nucleic acids which have been substantially purified from other components which naturally accompany the nucleic acid, i.e., RNA or DNA or proteins, which naturally accompany it in the cell. The term therefore includes, for example, a recombinant DNA which is incorporated into a vector, into an autonomously replicating plasmid or virus, or into the genomic DNA of a

prokaryote or eukaryote, or which exists as a separate molecule (i.e., as a cDNA or a genomic or cDNA fragment produced by PCR or restriction enzyme digestion) independent of other sequences. It also includes a recombinant DNA which is part of a hybrid gene encoding additional polypeptide sequence.

In the context of the present invention, the following abbreviations for the commonly occurring nucleic acid bases are used. "A" refers to adenosine, "C" refers to cytosine, "G" refers to guanosine, "T" refers to thymidine, and "U" refers to uridine.

The term "polynucleotide" as used herein is defined as a chain of nucleotides. Furthermore, nucleic acids are polymers of nucleotides. Thus, nucleic acids and polynucleotides as used herein are interchangeable. One skilled in the art has the general knowledge that nucleic acids are polynucleotides, which can be hydrolyzed into the monomeric "nucleotides." The monomeric nucleotides can be hydrolyzed into nucleosides. As used herein polynucleotides include, but are not limited to, all nucleic acid sequences which are obtained by any means available in the art, including, without limitation, recombinant means, i.e., the cloning of nucleic acid sequences from a recombinant library or a cell genome, using ordinary cloning technology and PCR, and the like, and by synthetic means.

As used herein, the terms "peptide," "polypeptide," and "protein" are used interchangeably, and refer to a compound comprised of amino acid residues covalently linked by peptide bonds. A protein or peptide must contain at least two amino acids, and no limitation is placed on the maximum number of amino acids that can comprise a protein's or peptide's sequence. Polypeptides include any peptide or protein comprising two or more amino acids joined to each other by peptide bonds. As used herein, the term refers to both short chains, which also commonly are referred to in the art as peptides, oligopeptides and oligomers, for example, and to longer chains, which generally are referred to in the art as proteins, of which there are many types. "Polypeptides" include, for example, biologically active fragments, substantially homologous polypeptides, oligopeptides, homodimers, heterodimers, variants of polypeptides, modified polypeptides, derivatives, analogs, fusion proteins, among others. The polypeptides include natural peptides, recombinant peptides, synthetic peptides, or a combination thereof.

As used herein, "conjugated" refers to covalent attachment of one molecule to a second molecule.

"Variant" as the term is used herein, is a nucleic acid sequence or an amino acid sequence that differs in sequence from a reference nucleic acid sequence or amino acid sequence respectively, but retains essential biological properties of the reference molecule. Changes in the sequence of a nucleic acid variant may not alter the amino acid sequence of a peptide encoded by the reference nucleic acid, or may result in amino acid substitutions, additions, deletions, fusions and truncations. Changes in the sequence of peptide variants are typically limited or conservative, so that the sequences of the reference peptide and the variant are closely similar overall and, in many regions, identical. A variant and reference peptide can differ in amino acid sequence by one or more substitutions, additions, deletions in any combination. A variant of a nucleic acid or peptide can be a naturally occurring such as an allelic variant, or can be a variant that is not known to occur naturally. Non-naturally occurring variants of nucleic acids and peptides may be made by mutagenesis techniques or by direct synthesis. In some embodiments, the variant sequence is at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, identical to the reference sequence.

Ranges: throughout this disclosure, various aspects of the invention can be presented in a range format. It should be understood that the description in range format is merely for convenience and brevity and should not be construed as an inflexible limitation on the scope of the invention. Accordingly, the description of a range should be considered to have specifically disclosed all the possible subranges as well as individual numerical values within that range. For example, description of a range such as from 1 to 6 should be considered to have specifically disclosed subranges such as from 1 to 3, from 1 to 4, from 1 to 5, from 2 to 4, from 2 to 6, from 3 to 6 etc., as well as individual numbers within that range, for example, 1, 2, 2.7, 3, 4, 5, 5.3, and 6. This applies regardless of the breadth of the range.

Description

The present invention is based in part on the discovery that PKCα activation results in inhibition of autophagy and that this is meditated through upregulation of miR-129-2. The present invention is further based in part on the discovery that miR-129-2 functions as an inhibitor of PPAR-γ coactivator 1α (PGC-1α) which regulates mitochondrial function and cell viability.

The present invention relates generally to compositions and methods for modulating autophagy. In one embodiment, the present invention provides compositions and methods for increasing the amount of autophagy in a subject, cell, tissue or organ in need thereof. In one embodiment, the present invention provides compositions and methods for decreasing or inhibiting the level or activity of a negative regulator of autophagy, thereby increasing autophagy. In one embodiment, the present invention provides compositions and methods for increasing the level or activity of a regulator of mitochondrial function, thereby increasing autophagy.

In one embodiment, the present invention is directed to methods and compositions for treatment, inhibition, prevention, or reduction of hyperglycemia-induced neural tube defects, or a disease or disorder associated with hyperglycemia-induced neural tube defects. In various embodiments, the present invention is directed to methods and compositions for treatment, inhibition, prevention, or reduction of a range of diseases including, but not limited to, spina bifida, exencephaly, craniorachischisis, microcephaly, chiari malformation, anencephaly and fetal or infant death.

In various embodiments, the compositions of the invention comprises a modulator of autophagocytosis, the level or activity of a regulator of autophagocytosis or the level or activity of a modulator of mitochondrial function. In one embodiment, the composition comprises a modulator of one or more of PGC-1α, PPARγ, PKCα and miR-129-2. In one embodiment, the modulator of PGC-1α, PPARγ or Sirtuin-1 increases the expression, activity, or both of PGC-1α, PPARγ or Sirtuin-1. In one embodiment, the modulator of PKCα or miR-129-2 inhibits the expression, activity, or both of PKCα or miR-129-2.

In one embodiment, the present invention provides compositions for treating or preventing a disease or disorder associated with decreased autophagy. In one

embodiment, the present invention comprises a composition for treating or preventing a hypoglycemia-induced neural tube defect, wherein the composition comprises a modulator of autophagocytosis.

Compositions

In various embodiments, the present invention includes compositions for modulating one or more of autophagy and mitochondrial function in a subject, a cell, a tissue, or an organ in need thereof. In various embodiments, the compositions of the invention modulates the level of autophagocytosis, the level or activity of a regulator of autophagy or the level of mitochondrial function (e.g., cellular respiration) in a subject, a cell, a tissue, or an organ in need thereof.

In one embodiment, a negative regulator of autophagy is PKCα. Therefore in one embodiment, the composition comprises a modulator of the expression or activity of PKCα. For example, in one embodiment, the modulator decreases the expression or activity of PKCα. In one embodiment, the compositions of the invention modulates the level of PKCα, the amount of mRNA encoding for PKCα, the activity of PKCα, or a combination thereof.

In one embodiment, a negative regulator of autophagy is miR-129-2. Therefore in one embodiment, the compositions of the invention modulates the level of miR-129-2, the activity of a miR-129-2, or a combination thereof.

In one embodiment, a regulator of mitochondrial function is PGC-1α, PPARγ or Sirtuin-1. Therefore in one embodiment, the composition comprises a modulator of the expression or activity of PGC-1α, PPARγ or a combination thereof. For example, in one embodiment, the modulator increases the expression or activity of PGC-1α, PPARγ or a combination thereof. In one embodiment, the compositions of the invention modulates the level of PGC-1α, PPARγ or Sirtuin-1, the amount of mRNA encoding for PGC-1α, PPARγ or Sirtuin-1, the activity of PGC-1α, PPARγ or Sirtuin-1, or a combination thereof.

Modulation of a gene, or gene product, can be assessed using a wide variety of methods, including those disclosed herein, as well as methods known in the art or to be developed in the future. That is, the person having ordinary skill in the art would

appreciate, based upon the disclosure provided herein, that modulating the level or activity of a gene, or gene product, can be readily assessed using methods that assess the level of a nucleic acid encoding a gene product (e.g., mRNA), the level of polypeptide gene product present in a biological sample, the activity of polypeptide gene product present in a biological sample, or combinations thereof.

The modulator compositions and methods of the invention that modulate the level or activity of a gene, or gene product, include, but should not be construed as being limited to, a chemical compound, a protein, a peptide, a peptidomimetic, an antibody, a ribozyme, an organic compound, an inorganic compound, a small molecule, a nucleic acid, a vector, an antisense nucleic acid molecule (e.g., siRNA, miRNA, etc.), or combinations thereof. One of skill in the art would readily appreciate, based on the disclosure provided herein, that a modulator composition encompasses a chemical compound that modulates the level or activity of a gene, or gene product. Additionally, a modulator composition encompasses a chemically modified compound, and derivatives, as is well known to one of skill in the chemical arts.

When the modulator of the invention is a small molecule, a small molecule agonist or antagonist may be obtained using standard methods known to the skilled artisan. Such methods include chemical organic synthesis or biological means. Biological means include purification from a biological source, recombinant synthesis and in vitro translation systems, using methods well known in the art.

Combinatorial libraries of molecularly diverse chemical compounds potentially useful in treating a variety of diseases and conditions are well known in the art as are method of making the libraries. The method may use a variety of techniques well-known to the skilled artisan including solid phase synthesis, solution methods, parallel synthesis of single compounds, synthesis of chemical mixtures, rigid core structures, flexible linear sequences, deconvolution strategies, tagging techniques, and generating unbiased molecular landscapes for lead discovery vs. biased structures for lead development.

In one embodiment, the modulator composition of the present invention is an antagonist, which decreases the expression, activity, or biological function of a gene or

gene product. For example, in certain embodiments, the modulator of the present invention is an antagonist of PKCα or miR-129-2.

In one embodiment, the modulator composition of the present invention is an agonist, which increases the expression, activity, or biological function of a gene or gene product. For example, in certain embodiments, the modulator of the present invention is an agonist of PGC-1α, PPARγ or Sirtuin-1.

Further, one of skill in the art would, when equipped with this disclosure and the methods exemplified herein, appreciate that modulators include such modulators as discovered in the future, as can be identified by well-known criteria in the art of pharmacology, such as the physiological results of modulation of the genes, and gene products, as described in detail herein and/or as known in the art. Therefore, the present invention is not limited in any way to any particular modulator composition as exemplified or disclosed herein; rather, the invention encompasses those modulator compositions that would be understood by the person having ordinary skill in the art to be useful as are known in the art and as are discovered in the future.

Further methods of identifying and producing modulator compositions are well known to those of ordinary skill in the art. Alternatively, a modulator can be synthesized chemically. Further, the person having ordinary skill in the art would appreciate, based upon the teachings provided herein, that a modulator composition can be obtained from a recombinant organism. Compositions and methods for chemically synthesizing modulators and for obtaining them from natural sources are well known in the art and are described in the art.

One of skill in the art will appreciate that a modulator can be administered as an organic compound, an inorganic compound, a small molecule, a polypeptide, a peptide, an antibody, a nucleic acid construct encoding a protein, an antisense nucleic acid, a nucleic acid construct encoding an antisense nucleic acid, or combinations thereof. Numerous vectors and other compositions and methods are well known for administering a protein or a nucleic acid construct encoding a protein to cells or tissues. Therefore, the invention includes a peptide or a nucleic acid encoding a peptide that is modulator of a gene, or gene product. For example, the invention includes a peptide or a nucleic acid encoding a peptide that comprises PGC-1α, PPARγ or Sirtuin-1, one or more functional

PGC-1α, PPARγ or Sirtuin-1 peptides or a combination thereof. (Sambrook et al., 2001, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York; Ausubel et al., 1997, Current Protocols in Molecular Biology, John Wiley & Sons, New York).

Activators

In various embodiments, the composition for treating a disease or disorder associated with decreased levels of autophagy comprises an activator of autophagy or autophagocytosis.

It will be understood by one skilled in the art, based upon the disclosure provided herein, that PGC-1α and PPARγ proteins function as activators of autophagy and mitochondrial function. Therefore, in various embodiments, the invention provides compositions comprising PGC-1α, PPARγ or Sirtuin-1 proteins, activators of PGC-1α, PPARγ or Sirtuin-1, functional PGC-1α, PPARγ or Sirtuin-1 peptides, and PGC-1α, PPARγ or Sirtuin-1 peptidomimetics. In one embodiment, the activators of the invention results in an increase in expression of PGC-1α, PPARγ or Sirtuin-1, including transcription, translation, or both. In one embodiment, the activators of the invention results in an increase in at least one activity of PGC-1α, PPARγ or Sirtuin-1. Thus, increasing the level or activity of PGC-1α, PPARγ or Sirtuin-1 includes, but is not limited to, increasing the amount of PGC-1α, PPARγ or Sirtuin-1 protein, increasing transcription, translation, or both, of a nucleic acid encoding PGC-1α, PPARγ or Sirtuin-1; and it also includes increasing any activity of PGC-1α, PPARγ or Sirtuin-1 as well.

Thus, the present invention relates to the prevention and treatment of a disease or disorder associated with decreased autophagocytosis by administration of a PGC-1α, PPARγ or Sirtuin-1 polypeptide, a recombinant PGC-1α, PPARγ or Sirtuin-1 polypeptide, an active PGC-1α, PPARγ or Sirtuin-1 polypeptide fragment, or an activator of PGC-1α, PPARγ or Sirtuin-1 expression or activity.

One of skill in the art will appreciate that an activator can be administered as a small molecule chemical, a protein, a nucleic acid construct encoding a protein, or combinations thereof. Numerous vectors and other compositions and methods are well known for administering a protein or a nucleic acid construct encoding a protein to cells

or tissues. Therefore, the invention includes a method of administering a protein or a nucleic acid encoding a protein that is an activator of autophagocytosis.

One of skill in the art will realize that diminishing the amount or activity of a molecule that itself diminishes the amount of autophagocytosis can serve to increase the amount or activity of autophagocytosis. Any inhibitor of a negative regulator of autophagocytosis is encompassed in the invention. As a non-limiting example, antisense oligonucleotide molecules are described as a form of inhibiting a regulator of autophagocytosis in order to increase the amount autophagocytosis. Antisense oligonucleotides are DNA or RNA molecules that are complementary to some portion of a mRNA molecule. When present in a cell, antisense oligonucleotides hybridize to an existing mRNA molecule and inhibit translation into a gene product. Inhibiting the expression of a gene using an antisense oligonucleotide is well known in the art (Marcus-Sekura, 1988, Anal. Biochem. 172:289), as are methods of expressing an antisense oligonucleotide in a cell (Inoue, U.S. Pat. No. 5,190,931). The methods of the invention include the use of antisense oligonucleotide to diminish the amount of a molecule that causes a decrease in the amount of autophagocytosis, thereby increasing the amount of autophagocytosis. Contemplated in the present invention are antisense oligonucleotides that are synthesized and provided to the cell by way of methods well known to those of ordinary skill in the art. As an example, an antisense oligonucleotide can be synthesized to be between about 10 and about 100, more preferably between about 15 and about 50 nucleotides long. The synthesis of nucleic acid molecules is well known in the art, as is the synthesis of modified antisense oligonucleotides to improve biological activity in comparison to unmodified antisense oligonucleotides (Tullis, 1991, U.S. Pat. No. 5,023,243).

Similarly, the expression of a gene may be inhibited by the hybridization of an antisense molecule to a promoter or other regulatory element of a gene, thereby affecting the transcription of the gene. Methods for the identification of a promoter or other regulatory element that interacts with a gene of interest are well known in the art, and include such methods as the yeast two hybrid system (Bartel and Fields, eds., In: The Yeast Two Hybrid System, Oxford University Press, Cary, N.C.).

Alternatively, inhibition of a gene expressing a protein that diminishes the level of autophagocytosis can be accomplished through the use of an siRNA, shRNA, antisense oligonucleotide or ribozyme. Given the nucleotide sequence of the molecule, one of ordinary skill in the art could synthesize an antisense oligonucleotide or ribozyme without undue experimentation, provided with the disclosure and references incorporated herein.

In one embodiment, an activator of the invention comprises an inhibitor of PKCα. In one embodiment, an inhibitor of PKCα is an antisense oligonucleotide capable of specifically binding to an mRNA molecule encoding PKCα.

In one embodiment, an activator of the invention comprises an inhibitor of miR-129-2. In one embodiment, an inhibitor of miR-129-2 is an antisense oligonucleotide capable of specifically binding to miR-129-2 (e.g, an antagomiR).

Epoxyeicosatrienoic acids (EETs)

In certain embodiments, the activator of autophagy of the invention is an EET or an analog thereof. EETs include, but are not limited to, 5,6-EET, 8,9-EET, 11,12-EET, 14,15-EET, epoxyeicosatrienoic acid analogue EET-A, and (±)14(15)-EET. In one embodiment, the EET or analog thereof is formulated with a pharmaceutically-acceptable carrier.

As used herein, the term "pharmaceutically-acceptable carrier" means a chemical composition with which an appropriate polypeptide, a recombinant polypeptide, an active polypeptide fragment, an organic compound, an inorganic compound, a small molecule or activator, may be combined and which, following the combination, can be used to administer the appropriate polypeptide, recombinant polypeptide, active polypeptide fragment, organic compound, inorganic compound, small molecule or activator to a subject.

PGC-1α Activators

In certain embodiments, the invention provides compositions and methods for activating PGC-1α. PGC-1α agonists include, but are not limited to, ZLN005, telmisartan and fenofibrate.

The invention encompasses administration of a PGC-1α polypeptide, a recombinant PGC-1α polypeptide, an active PGC-1α polypeptide fragment, an organic compound, an inorganic compound, a small molecule or PGC-1α activator to practice the methods of the invention; the skilled artisan would understand, based on the disclosure provided herein, how to formulate and administer the appropriate a PGC-1α polypeptide, a recombinant PGC-1α polypeptide, an active PGC-1α polypeptide fragment, an organic compound, an inorganic compound, a small molecule or PGC-1α activator to a subject. However, the present invention is not limited to any particular method of administration or treatment regimen. This is especially true where it would be appreciated by one skilled in the art, equipped with the disclosure provided herein, including the reduction to practice using an art-recognized model of a disease, that methods of administering a PGC-1α polypeptide, a recombinant PGC-1α polypeptide, an active PGC-1α polypeptide fragment, an organic compound, an inorganic compound, a small molecule or PGC-1α activator can be determined by one of skill in the pharmacological arts. In one embodiment, the PGC-1α agonist is formulated with a pharmaceutically-acceptable carrier.

PPARγ Activators

In certain embodiments, the invention provides compositions and methods for activating PPARγ. PPARγ agonists include, but are not limited to, thiazolidinediones, rosiglitazone, pioglitazone, honokiol, amorfrutin 1, amorfrutin B, amorphastilbol, roscovitine, aleglitazar, muraglitazar, saroglitazar and tesaglitazar.

The invention encompasses administration of a PPARγ polypeptide, a recombinant PPARγ polypeptide, an active PPARγ polypeptide fragment, an organic compound, an inorganic compound, a small molecule or PPARγ activator to practice the methods of the invention; the skilled artisan would understand, based on the disclosure provided herein, how to formulate and administer the appropriate a PPARγ polypeptide, a recombinant PPARγ polypeptide, an active PPARγ polypeptide fragment, an organic compound, an inorganic compound, a small molecule or PPARγ activator to a subject. However, the present invention is not limited to any particular method of administration or treatment regimen. This is especially true where it would be appreciated by one skilled

in the art, equipped with the disclosure provided herein, including the reduction to practice using an art-recognized model of a disease, that methods of administering a PPARγ polypeptide, a recombinant PPARγ polypeptide, an active PPARγ polypeptide fragment, an organic compound, an inorganic compound, a small molecule or PPARγ activator can be determined by one of skill in the pharmacological arts. In one embodiment, the PPARγ agonist is formulated with a pharmaceutically-acceptable carrier.

Sirtuin Activators

Sirtuin activators can decrease PGC-1α acetylation and, thus, increase PGC-1α and PPARγ activity. Therefore, in certain embodiments, the invention provides compositions and methods for activating a Sirtuin. In one embodiment, a Sirtuin is Sirtuin-1. Sirtuin agonists include, but are not limited to, Resveratrol, Resveratrol modified compounds with improved bioavailability, resVida, Lonevinex, SRT501, Pterostilbene, agonists of sirtuins unrelated to resveratrol including but not limited to SRT1720, SRT2104, SRT2379, berberine, acetylsalicylic acid, Metformin, AICAR, AZD-769662 oxaloacetate, and inhibitors of mTOR, including but not limited to rapamycin.

The invention encompasses administration of a Sirtuin polypeptide, a recombinant Sirtuin polypeptide, an active Sirtuin polypeptide fragment, an organic compound, an inorganic compound, a small molecule or Sirtuin activator to practice the methods of the invention; the skilled artisan would understand, based on the disclosure provided herein, how to formulate and administer the appropriate a Sirtuin polypeptide, a recombinant Sirtuin polypeptide, an active Sirtuin polypeptide fragment, an organic compound, an inorganic compound, a small molecule or Sirtuin activator to a subject. However, the present invention is not limited to any particular method of administration or treatment regimen. This is especially true where it would be appreciated by one skilled in the art, equipped with the disclosure provided herein, including the reduction to practice using an art-recognized model of a disease, that methods of administering a Sirtuin polypeptide, a recombinant Sirtuin polypeptide, an active Sirtuin polypeptide fragment, an organic compound, an inorganic compound, a small molecule or Sirtuin

activator can be determined by one of skill in the pharmacological arts. In one embodiment, the Sirtuin agonist is formulated with a pharmaceutically-acceptable carrier.

Peptides

In one embodiment, the modulator of the present invention comprises an active PGC-1α, PPARγ or Sirtuin-1 polypeptide, or fragment thereof. For example, in one embodiment, a peptide of the composition comprises an amino acid sequence of PGC-1α, PPARγ or Sirtuin-1. In certain embodiments, the peptide comprises a functional fragment of PGC-1α, PPARγ or Sirtuin-1.

In one embodiment, the composition of the invention comprises a peptide, a fragment of a peptide, a homolog, a variant, a derivative or a salt of a peptide described herein. For example, in certain embodiments, the composition comprises a peptide comprising PGC-1α, PPARγ or Sirtuin-1 protein, a fragment of PGC-1α, PPARγ or Sirtuin-1, a homolog of PGC-1α, PPARγ or Sirtuin-1, a variant of PGC-1α, PPARγ or Sirtuin-1, a derivative of PGC-1α, PPARγ or Sirtuin-1, or a salt of PGC-1α, PPARγ or Sirtuin-1.

In certain embodiments, the peptide comprises a targeting domain, which targets the peptide to a desired location. For example, in certain embodiments, the targeting domain binds to a targeted cell, protein, or protein aggregate, thereby delivering the therapeutic peptide to a desired location. For example, in one embodiment, the targeting domain is directed to bind to a protein or protein aggregate associated with a disease or disorder, including but not limited to the proteins and protein aggregates of amyloid-beta, alpha-synuclein, tau, prions, SOD1, TDP-43, FUS, p53 mutants, and proteins associated with polyglutamine repeats, such as huntingtin, ataxins.

In certain embodiments, the targeting domain comprises a peptide, nucleic acid, small molecule, or the like, which has the ability to bind to the targeted cell, protein, or protein aggregate. For example, in one embodiment, the targeting domain comprises an antibody or antibody fragment which binds to a targeted cell, protein, or protein aggregate.

The peptide of the present invention may be made using chemical methods. For example, peptides can be synthesized by solid phase techniques (Roberge J

Y et al (1995) Science 269: 202-204), cleaved from the resin, and purified by preparative high performance liquid chromatography. Automated synthesis may be achieved, for example, using the ABI 431 A Peptide Synthesizer (Perkin Elmer) in accordance with the instructions provided by the manufacturer.

The peptide may alternatively be made by recombinant means or by cleavage from a longer polypeptide. The composition of a peptide may be confirmed by amino acid analysis or sequencing.

The variants of the peptides according to the present invention may be (i) one in which one or more of the amino acid residues are substituted with a conserved or non-conserved amino acid residue (preferably a conserved amino acid residue) and such substituted amino acid residue may or may not be one encoded by the genetic code, (ii) one in which there are one or more modified amino acid residues, e.g., residues that are modified by the attachment of substituent groups, (iii) one in which the peptide is an alternative splice variant of the peptide of the present invention, (iv) fragments of the peptides and/or (v) one in which the peptide is fused with another peptide, such as a leader or secretory sequence or a sequence which is employed for purification (for example, His-tag) or for detection (for example, Sv5 epitope tag). The fragments include peptides generated via proteolytic cleavage (including multi-site proteolysis) of an original sequence. Variants may be post-translationally, or chemically modified. Such variants are deemed to be within the scope of those skilled in the art from the teaching herein.

The peptides of the invention can be post-translationally modified. For example, post-translational modifications that fall within the scope of the present invention include signal peptide cleavage, glycosylation, acetylation, isoprenylation, proteolysis, myristoylation, protein folding and proteolytic processing, etc. Some modifications or processing events require introduction of additional biological machinery. For example, processing events, such as signal peptide cleavage and core glycosylation, are examined by adding canine microsomal membranes or Xenopus egg extracts (U.S. Pat. No. 6,103,489) to a standard translation reaction.

The peptides of the invention may include unnatural amino acids formed by post-translational modification or by introducing unnatural amino acids during

translation. A variety of approaches are available for introducing unnatural amino acids during protein translation. By way of example, special tRNAs, such as tRNAs which have suppressor properties, suppressor tRNAs, have been used in the process of site-directed non-native amino acid replacement (SNAAR). In SNAAR, a unique codon is required on the mRNA and the suppressor tRNA, acting to target a non-native amino acid to a unique site during the protein synthesis (described in WO90/05785). However, the suppressor tRNA must not be recognizable by the aminoacyl tRNA synthetases present in the protein translation system. In certain cases, a non-native amino acid can be formed after the tRNA molecule is aminoacylated using chemical reactions which specifically modify the native amino acid and do not significantly alter the functional activity of the aminoacylated tRNA. These reactions are referred to as post-aminoacylation modifications. For example, the epsilon-amino group of the lysine linked to its cognate tRNA ($tRNA_{LYS}$), could be modified with an amine specific photoaffinity label.

The peptides of the invention may be conjugated with other molecules, such as proteins, to prepare fusion proteins. This may be accomplished, for example, by the synthesis of N-terminal or C-terminal fusion proteins provided that the resulting fusion protein retains the functionality of the peptide of the invention.

Cyclic derivatives of the peptides the invention are also part of the present invention. Cyclization may allow the peptide to assume a more favorable conformation for association with other molecules. Cyclization may be achieved using techniques known in the art. For example, disulfide bonds may be formed between two appropriately spaced components having free sulfhydryl groups, or an amide bond may be formed between an amino group of one component and a carboxyl group of another component. Cyclization may also be achieved using an azobenzene-containing amino acid as described by Ulysse, L., et al., J. Am. Chem. Soc. 1995, 117, 8466-8467. The components that form the bonds may be side chains of amino acids, non-amino acid components or a combination of the two. In an embodiment of the invention, cyclic peptides may comprise a beta-turn in the right position. Beta-turns may be introduced into the peptides of the invention by adding the amino acids Pro-Gly at the right position.

It may be desirable to produce a cyclic peptide which is more flexible than the cyclic peptides containing peptide bond linkages as described above. A more flexible

peptide may be prepared by introducing cysteines at the right and left position of the peptide and forming a disulphide bridge between the two cysteines. The two cysteines are arranged so as not to deform the beta-sheet and turn. The peptide is more flexible as a result of the length of the disulfide linkage and the smaller number of hydrogen bonds in the beta-sheet portion. The relative flexibility of a cyclic peptide can be determined by molecular dynamics simulations.

The peptides of the invention may be converted into pharmaceutical salts by reacting with inorganic acids such as hydrochloric acid, sulfuric acid, hydrobromic acid, phosphoric acid, etc., or organic acids such as formic acid, acetic acid, propionic acid, glycolic acid, lactic acid, pyruvic acid, oxalic acid, succinic acid, malic acid, tartaric acid, citric acid, benzoic acid, salicylic acid, benezenesulfonic acid, and toluenesulfonic acids.

Peptides of the invention may also have modifications. Modifications (which do not normally alter primary sequence) include in vivo, or in vitro chemical derivatization of polypeptides, e.g., acetylation, or carboxylation. Also included are modifications of glycosylation, e.g., those made by modifying the glycosylation patterns of a polypeptide during its synthesis and processing or in further processing steps; e.g., by exposing the polypeptide to enzymes which affect glycosylation, e.g., mammalian glycosylating or deglycosylating enzymes. Also embraced are sequences which have phosphorylated amino acid residues, e.g., phosphotyrosine, phosphoserine, or phosphothreonine.

Also included are peptides which have been modified using ordinary molecular biological techniques so as to improve their resistance to proteolytic degradation or to optimize solubility properties or to render them more suitable as a therapeutic agent. Such variants include those containing residues other than naturally-occurring L-amino acids, e.g., D-amino acids or non-naturally-occurring synthetic amino acids. The peptides of the invention may further be conjugated to non-amino acid moieties that are useful in their therapeutic application. In particular, moieties that improve the stability, biological half-life, water solubility, and/or immunologic characteristics of the peptide are useful. A non-limiting example of such a moiety is polyethylene glycol (PEG).

Covalent attachment of biologically active compounds to water-soluble polymers is one method for alteration and control of biodistribution, pharmacokinetics, and often, toxicity for these compounds (Duncan et al., 1984, Adv. Polym. Sci. 57:53-101). Many water-soluble polymers have been used to achieve these effects, such as poly(sialic acid), dextran, poly(N-(2-hydroxypropyl)methacrylamide) (PHPMA), poly(N-vinylpyrrolidone) (PVP), poly(vinyl alcohol) (PVA), poly(ethylene glycol-co-propylene glycol), poly(N-acryloyl morpholine (PAcM), and poly(ethylene glycol) (PEG) (Powell, 1980, Polyethylene glycol. In R. L. Davidson (Ed.) Handbook of Water Soluble Gums and Resins. McGraw-Hill, New York, chapter 18). PEG possess an ideal set of properties: very low toxicity (Pang, 1993, J. Am. Coll. Toxicol. 12: 429-456) excellent solubility in aqueous solution (Powell, supra), low immunogenicity and antigenicity (Dreborg et al., 1990, Crit. Rev. Ther. Drug Carrier Syst. 6: 315-365). PEG-conjugated or "PEGylated" protein therapeutics, containing single or multiple chains of polyethylene glycol on the protein, have been described in the scientific literature (Clark et al., 1996, J. Biol. Chem. 271: 21969-21977; Hershfield, 1997, Biochemistry and immunology of poly(ethylene glycol)-modified adenosine deaminase (PEG-ADA). In J. M. Harris and S. Zalipsky (Eds) Poly(ethylene glycol): Chemistry and Biological Applications. American Chemical Society, Washington, D.C., p 145-154; Olson et al., 1997, Preparation and characterization of poly(ethylene glycol)ylated human growth hormone antagonist. In J. M. Harris and S. Zalipsky (Eds) Poly(ethylene glycol): Chemistry and Biological Applications. American Chemical Society, Washington, D.C., p 170-181).

A peptide of the invention may be synthesized by conventional techniques. For example, the peptides of the invention may be synthesized by chemical synthesis using solid phase peptide synthesis. These methods employ either solid or solution phase synthesis methods (see for example, J. M. Stewart, and J. D. Young, Solid Phase Peptide Synthesis, 2nd Ed., Pierce Chemical Co., Rockford Ill. (1984) and G. Barany and R. B. Merrifield, The Peptides: Analysis Synthesis, Biology editors E. Gross and J. Meienhofer Vol. 2 Academic Press, New York, 1980, pp. 3-254 for solid phase synthesis techniques; and M Bodansky, Principles of Peptide Synthesis, Springer-Verlag, Berlin 1984, and E. Gross and J. Meienhofer, Eds., The Peptides: Analysis, Synthesis, Biology, suprs, Vol 1, for classical solution synthesis.)

The peptides may be chemically synthesized by Merrifield-type solid phase peptide synthesis. This method may be routinely performed to yield peptides up to about 60-70 residues in length, and may, in some cases, be utilized to make peptides up to about 100 amino acids long. Larger peptides may also be generated synthetically via fragment condensation or native chemical ligation (Dawson et al., 2000, Ann. Rev. Biochem. 69:923-960). An advantage to the utilization of a synthetic peptide route is the ability to produce large amounts of peptides, even those that rarely occur naturally, with relatively high purities, i.e., purities sufficient for research, diagnostic or therapeutic purposes.

Solid phase peptide synthesis is described by Stewart et al. in Solid Phase Peptide Synthesis, 2nd Edition, 1984, Pierce Chemical Company, Rockford, Ill.; and Bodanszky and Bodanszky in The Practice of Peptide Synthesis, 1984, Springer-Verlag, New York. At the outset, a suitably protected amino acid residue is attached through its carboxyl group to a derivatized, insoluble polymeric support, such as cross-linked polystyrene or polyamide resin. "Suitably protected" refers to the presence of protecting groups on both the alpha-amino group of the amino acid, and on any side chain functional groups. Side chain protecting groups are generally stable to the solvents, reagents and reaction conditions used throughout the synthesis, and are removable under conditions which will not affect the final peptide product. Stepwise synthesis of the oligopeptide is carried out by the removal of the N-protecting group from the initial amino acid, and coupling thereto of the carboxyl end of the next amino acid in the sequence of the desired peptide. This amino acid is also suitably protected. The carboxyl of the incoming amino acid can be activated to react with the N-terminus of the support-bound amino acid by formation into a reactive group, such as formation into a carbodiimide, a symmetric acid anhydride, or an "active ester" group, such as hydroxybenzotriazole or pentafluorophenyl esters.

Examples of solid phase peptide synthesis methods include the BOC method which utilized tert-butyloxcarbonyl as the alpha-amino protecting group, and the FMOC method which utilizes 9-fluorenylmethyloxcarbonyl to protect the alpha-amino of the amino acid residues, both which methods are well-known by those of skill in the art.

Incorporation of N- and/or C-blocking groups may also be achieved using protocols conventional to solid phase peptide synthesis methods. For incorporation of C-terminal blocking groups, for example, synthesis of the desired peptide is typically performed using, as solid phase, a supporting resin that has been chemically modified so that cleavage from the resin results in a peptide having the desired C-terminal blocking group. To provide peptides in which the C-terminus bears a primary amino blocking group, for instance, synthesis is performed using a p-methylbenzhydrylamine (MBHA) resin, so that, when peptide synthesis is completed, treatment with hydrofluoric acid releases the desired C-terminally amidated peptide. Similarly, incorporation of an N-methylamine blocking group at the C-terminus is achieved using N-methylaminoethyl-derivatized DVB, resin, which upon HF treatment releases a peptide bearing an N-methylamidated C-terminus. Blockage of the C-terminus by esterification can also be achieved using conventional procedures. This entails use of resin/blocking group combination that permits release of side-chain peptide from the resin, to allow for subsequent reaction with the desired alcohol, to form the ester function. FMOC protecting group, in combination with DVB resin derivatized with methoxyalkoxybenzyl alcohol or equivalent linker, can be used for this purpose, with cleavage from the support being effected by TFA in dicholoromethane. Esterification of the suitably activated carboxyl function, e.g. with DCC, can then proceed by addition of the desired alcohol, followed by de-protection and isolation of the esterified peptide product.

The peptides of the invention may be prepared by standard chemical or biological means of peptide synthesis. Biological methods include, without limitation, expression of a nucleic acid encoding a peptide in a host cell or in an in vitro translation system.

Included in the invention are nucleic acid sequences that encode the peptide of the invention. In one embodiment, the invention includes nucleic acid sequences encoding the amino acid sequence of PGC-1α, PPARγ or Sirtuin-1.

Accordingly, subclones of a nucleic acid sequence encoding a peptide of the invention can be produced using conventional molecular genetic manipulation for subcloning gene fragments, such as described by Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Springs Laboratory, Cold Springs Harbor, New York (2012),

and Ausubel et al. (ed.), *Current Protocols in Molecular Biology*, John Wiley & Sons (New York, NY) (1999 and preceding editions), each of which is hereby incorporated by reference in its entirety. The subclones then are expressed *in vitro* or *in vivo* in bacterial cells to yield a smaller protein or polypeptide that can be tested for a particular activity.

Combined with certain formulations, such peptides can be effective intracellular agents. However, in order to increase the efficacy of such peptides, the one or more peptides of the invention can be provided a fusion peptide along with a second peptide which promotes "transcytosis", e.g., uptake of the peptide by cells. For example, in one embodiment, the peptide may comprise a cell-penetrating domain, for example a cell-penetrating peptide (CPP) to allow for the peptide to enter a cell. In one embodiment, the CPP is derived from HIV Tat.

To illustrate, the one or more peptides of the present invention can be provided as part of a fusion polypeptide with all or a fragment of the N-terminal domain of the HIV protein Tat, e.g., residues 1-72 of Tat or a smaller fragment thereof which can promote transcytosis. In one embodiment, the peptide comprises the protein transduction domain of HIV Tat. In other embodiments, the one or more peptides can be provided a fusion polypeptide with all or a portion of the antenopedia III protein. Other cell-penetrating domains that mediate uptake of the peptide are known in the art, and are equally applicable for use in a fusion peptide of the present invention.

Nucleic Acids

In one embodiment, the composition of the invention comprises one or isolated nucleic acids. For example, in one embodiment, the one or more isolated nucleic acids encodes an activator of the invention or a fragment or a variant thereof.

The nucleotide sequence of the isolated nucleic acids include both the DNA sequence that is transcribed into RNA and the RNA sequence that is translated into a polypeptide. According to other embodiments, the nucleotide sequences are inferred from the amino acid sequence of the peptides of the invention. As is known in the art several alternative nucleotide sequences are possible due to redundant codons, while retaining the biological activity of the translated peptides.

Thus, the invention encompasses expression vectors and methods for the introduction of exogenous DNA into cells with concomitant expression of the exogenous DNA in the cells such as those described, for example, in Sambrook et al. (2012, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York), and in Ausubel et al. (1997, Current Protocols in Molecular Biology, John Wiley & Sons, New York).

The desired nucleic acid encoding PGC-1α, PPARγ or Sirtuin-1 can be cloned into a number of types of vectors. However, the present invention should not be construed to be limited to any particular vector. Instead, the present invention should be construed to encompass a wide plethora of vectors which are readily available and/or well-known in the art. For example, a desired polynucleotide of the invention can be cloned into a vector including, but not limited to a plasmid, a phagemid, a phage derivative, an animal virus, and a cosmid. Vectors of particular interest include expression vectors, replication vectors, probe generation vectors, and sequencing vectors.

In specific embodiments, the expression vector is selected from the group consisting of a viral vector, a bacterial vector and a mammalian cell vector. Numerous expression vector systems exist that comprise at least a part or all of the compositions discussed above. Prokaryote- and/or eukaryote-vector based systems can be employed for use with the present invention to produce polynucleotides, or their cognate polypeptides. Many such systems are commercially and widely available.

Further, the expression vector may be provided to a cell in the form of a viral vector. Viral vector technology is well known in the art and is described, for example, in Sambrook et al. (2012), and in Ausubel et al. (1997), and in other virology and molecular biology manuals. Viruses, which are useful as vectors include, but are not limited to, retroviruses, adenoviruses, adeno-associated viruses, herpes viruses, and lentiviruses. In general, a suitable vector contains an origin of replication functional in at least one organism, a promoter sequence, convenient restriction endonuclease sites, and one or more selectable markers. (See, e.g., WO 01/96584; WO 01/29058; and U.S. Pat. No. 6,326,193.

A number of viral based systems have been developed for gene transfer into mammalian cells. For example, retroviruses provide a convenient platform for gene

delivery systems. A selected gene can be inserted into a vector and packaged in retroviral particles using techniques known in the art. The recombinant virus can then be isolated and delivered to cells of the subject either *in vivo* or *ex vivo*. A number of retroviral systems are known in the art. In some embodiments, adenovirus vectors are used. A number of adenovirus vectors are known in the art. In one embodiment, lentivirus vectors are used.

For example, vectors derived from retroviruses such as the lentivirus are suitable tools to achieve long-term gene transfer since they allow long-term, stable integration of a transgene and its propagation in daughter cells. Lentiviral vectors have the added advantage over vectors derived from onco-retroviruses such as murine leukemia viruses in that they can transduce non-proliferating cells, such as hepatocytes. They also have the added advantage of low immunogenicity. In a preferred embodiment, the composition includes a vector derived from an adeno-associated virus (AAV). Adeno-associated viral (AAV) vectors have become powerful gene delivery tools for the treatment of various disorders. AAV vectors possess a number of features that render them ideally suited for gene therapy, including a lack of pathogenicity, minimal immunogenicity, and the ability to transduce postmitotic cells in a stable and efficient manner. Expression of a particular gene contained within an AAV vector can be specifically targeted to one or more types of cells by choosing the appropriate combination of AAV serotype, promoter, and delivery method

In one embodiment, the encoding sequence is contained within an AAV vector. More than 30 naturally occurring serotypes of AAV are available. Many natural variants in the AAV capsid exist, allowing identification and use of an AAV with properties specifically suited for skeletal muscle. AAV viruses may be engineered using conventional molecular biology techniques, making it possible to optimize these particles for cell specific delivery of nucleic acid sequences, for minimizing immunogenicity, for tuning stability and particle lifetime, for efficient degradation, for accurate delivery to the nucleus, etc.

Thus, an increase in expression of PGC-1α, PPARγ or Sirtuin-1 can be achieved by delivering a recombinantly engineered AAV or artificial AAV that contains one or more encoding sequences. The use of AAVs is a common mode of exogenous

delivery of DNA as it is relatively non-toxic, provides efficient gene transfer, and can be easily optimized for specific purposes. Exemplary AAV serotypes include, but is not limited to AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8 and AAV9.

Desirable AAV fragments for assembly into vectors include the cap proteins, including the vp1, vp2, vp3 and hypervariable regions, the rep proteins, including rep 78, rep 68, rep 52, and rep 40, and the sequences encoding these proteins. These fragments may be readily utilized in a variety of vector systems and host cells. Such fragments may be used alone, in combination with other AAV serotype sequences or fragments, or in combination with elements from other AAV or non-AAV viral sequences. As used herein, artificial AAV serotypes include, without limitation, AAV with a non-naturally occurring capsid protein. Such an artificial capsid may be generated by any suitable technique, using a selected AAV sequence (e.g., a fragment of a vp1 capsid protein) in combination with heterologous sequences which may be obtained from a different selected AAV serotype, non-contiguous portions of the same AAV serotype, from a non-AAV viral source, or from a non-viral source. An artificial AAV serotype may be, without limitation, a chimeric AAV capsid, a recombinant AAV capsid, or a "humanized" AAV capsid. Thus exemplary AAVs, or artificial AAVs, suitable for expression of PGC-1α, PPARγ or Sirtuin-1, include AAV2/8 (see U.S. Pat. No. 7,282,199), AAV2/5 (available from the National Institutes of Health), AAV2/9 (International Patent Publication No. WO2005/033321), AAV2/6 (U.S. Pat. No. 6,156,303), and AAVrh8 (International Patent Publication No. WO2003/042397), among others.

For expression of the desired polynucleotide, at least one module in each promoter functions to position the start site for RNA synthesis. The best known example of this is the TATA box, but in some promoters lacking a TATA box, such as the promoter for the mammalian terminal deoxynucleotidyl transferase gene and the promoter for the SV40 genes, a discrete element overlying the start site itself helps to fix the place of initiation.

Additional promoter elements, i.e., enhancers, regulate the frequency of transcriptional initiation. Typically, these are located in the region 30-110 bp upstream of the start site, although a number of promoters have recently been shown to contain

functional elements downstream of the start site as well. The spacing between promoter elements frequently is flexible, so that promoter function is preserved when elements are inverted or moved relative to one another. In the thymidine kinase (tk) promoter, the spacing between promoter elements can be increased to 50 bp apart before activity begins to decline. Depending on the promoter, it appears that individual elements can function either co-operatively or independently to activate transcription.

A promoter may be one naturally associated with a gene or polynucleotide sequence, as may be obtained by isolating the 5′ non-coding sequences located upstream of the coding segment and/or exon. Such a promoter can be referred to as "endogenous." Similarly, an enhancer may be one naturally associated with a polynucleotide sequence, located either downstream or upstream of that sequence. Alternatively, certain advantages will be gained by positioning the coding polynucleotide segment under the control of a recombinant or heterologous promoter, which refers to a promoter that is not normally associated with a polynucleotide sequence in its natural environment. A recombinant or heterologous enhancer refers also to an enhancer not normally associated with a polynucleotide sequence in its natural environment. Such promoters or enhancers may include promoters or enhancers of other genes, and promoters or enhancers isolated from any other prokaryotic, viral, or eukaryotic cell, and promoters or enhancers not "naturally occurring," *i.e.,* containing different elements of different transcriptional regulatory regions, and/or mutations that alter expression. In addition to producing nucleic acid sequences of promoters and enhancers synthetically, sequences may be produced using recombinant cloning and/or nucleic acid amplification technology, including PCR™, in connection with the compositions disclosed herein (U.S. Patent 4,683,202, U.S. Patent 5,928,906). Furthermore, it is contemplated the control sequences that direct transcription and/or expression of sequences within non-nuclear organelles such as mitochondria, chloroplasts, and the like, can be employed as well.

Naturally, it will be important to employ a promoter and/or enhancer that effectively directs the expression of the DNA segment in the cell type, organelle, and organism chosen for expression. Those of skill in the art of molecular biology generally know how to use promoters, enhancers, and cell type combinations for protein expression, for example, see Sambrook et al. (2012). The promoters employed may be

constitutive, tissue-specific, inducible, and/or useful under the appropriate conditions to direct high level expression of the introduced DNA segment, such as is advantageous in the large-scale production of recombinant proteins and/or peptides. The promoter may be heterologous or endogenous.

In order to assess the expression of the desired polynucleotide, the expression vector to be introduced into a cell can also contain either a selectable marker gene or a reporter gene or both to facilitate identification and selection of expressing cells from the population of cells sought to be transfected or infected through viral vectors. In other embodiments, the selectable marker may be carried on a separate piece of DNA and used in a co-transfection procedure. Both selectable markers and reporter genes may be flanked with appropriate regulatory sequences to enable expression in the host cells. Useful selectable markers are known in the art and include, for example, antibiotic-resistance genes, such as neo and the like.

Reporter genes are used for identifying potentially transfected cells and for evaluating the functionality of regulatory sequences. Reporter genes that encode for easily assayable proteins are well known in the art. In general, a reporter gene is a gene that is not present in or expressed by the recipient organism or tissue and that encodes a protein whose expression is manifested by some easily detectable property, e.g., enzymatic activity. Expression of the reporter gene is assayed at a suitable time after the DNA has been introduced into the recipient cells.

Suitable reporter genes may include genes encoding luciferase, beta-galactosidase, chloramphenicol acetyl transferase, secreted alkaline phosphatase, or the green fluorescent protein gene (see, e.g., Ui-Tei et al., 2000 FEBS Lett. 479:79-82). Suitable expression systems are well known and may be prepared using well known techniques or obtained commercially. Internal deletion constructs may be generated using unique internal restriction sites or by partial digestion of non-unique restriction sites. Constructs may then be transfected into cells that display high levels of siRNA polynucleotide and/or polypeptide expression. In general, the construct with the minimal 5' flanking region showing the highest level of expression of reporter gene is identified as the promoter. Such promoter regions may be linked to a reporter gene and used to evaluate agents for the ability to modulate promoter-driven transcription.

In the context of an expression vector, the vector can be readily introduced into a host cell, e.g., mammalian, bacterial, yeast or insect cell by any method in the art. For example, the expression vector can be transferred into a host cell by physical, chemical or biological means.

Physical methods for introducing a polynucleotide into a host cell include calcium phosphate precipitation, lipofection, particle bombardment, microinjection, electroporation, and the like. Methods for producing cells comprising vectors and/or exogenous nucleic acids are well-known in the art. See, for example, Sambrook et al. (2012, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York), and in Ausubel et al. (1997, Current Protocols in Molecular Biology, John Wiley & Sons, New York).

Biological methods for introducing a polynucleotide of interest into a host cell include the use of DNA and RNA vectors. Viral vectors, and especially retroviral vectors, have become the most widely used method for inserting genes into mammalian, e.g., human cells. Other viral vectors can be derived from lentivirus, poxviruses, herpes simplex virus I, adenoviruses and adeno-associated viruses, and the like. See, for example, U.S. Pat. Nos. 5,350,674 and 5,585,362.

Chemical means for introducing a polynucleotide into a host cell include colloidal dispersion systems, such as macromolecule complexes, nanocapsules, microspheres, beads, and lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, and liposomes. A preferred colloidal system for use as a delivery vehicle *in vitro* and *in vivo* is a liposome (i.e., an artificial membrane vesicle). The preparation and use of such systems is well known in the art.

Regardless of the method used to introduce exogenous nucleic acids into a host cell, in order to confirm the presence of the recombinant DNA sequence in the host cell, a variety of assays may be performed. Such assays include, for example, "molecular biological" assays well known to those of skill in the art, such as Southern and Northern blotting, RT-PCR and PCR; "biochemical" assays, such as detecting the presence or absence of a particular peptide, e.g., by immunological means (ELISAs and Western blots) or by assays described herein to identify agents falling within the scope of the invention.

Any DNA vector or delivery vehicle can be utilized to transfer the desired polynucleotide to a cell *in vitro* or *in vivo*. In the case where a non-viral delivery system is utilized, a preferred delivery vehicle is a liposome. The above-mentioned delivery systems and protocols therefore can be found in Gene Targeting Protocols, 2ed., pp 1-35 (2002) and Gene Transfer and Expression Protocols, Vol. 7, Murray ed., pp 81-89 (1991).

"Liposome" is a generic term encompassing a variety of single and multilamellar lipid vehicles formed by the generation of enclosed lipid bilayers or aggregates. Liposomes may be characterized as having vesicular structures with a phospholipid bilayer membrane and an inner aqueous medium. Multilamellar liposomes have multiple lipid layers separated by aqueous medium. They form spontaneously when phospholipids are suspended in an excess of aqueous solution. The lipid components undergo self-rearrangement before the formation of closed structures and entrap water and dissolved solutes between the lipid bilayers. However, the present invention also encompasses compositions that have different structures in solution than the normal vesicular structure. For example, the lipids may assume a micellar structure or merely exist as nonuniform aggregates of lipid molecules. Also contemplated are lipofectamine-nucleic acid complexes.

In one embodiment, the composition of the invention comprises in vitro transcribed (IVT) RNA encoding an activator of the invention. In one embodiment, an IVT RNA can be introduced to a cell as a form of transient transfection. The RNA is produced by in vitro transcription using a plasmid DNA template generated synthetically. DNA of interest from any source can be directly converted by PCR into a template for in vitro mRNA synthesis using appropriate primers and RNA polymerase. The source of the DNA can be, for example, genomic DNA, plasmid DNA, phage DNA, cDNA, synthetic DNA sequence or any other appropriate source of DNA.

In one embodiment, the DNA to be used for PCR contains an open reading frame. The DNA can be from a naturally occurring DNA sequence from the genome of an organism. In one embodiment, the DNA is a full length gene of interest of a portion of a gene. The gene can include some or all of the 5' and/or 3' untranslated regions (UTRs). The gene can include exons and introns. In one embodiment, the DNA to be used for PCR is a human gene. In another embodiment, the DNA to be used for PCR is a human

gene including the 5' and 3' UTRs. The DNA can alternatively be an artificial DNA sequence that is not normally expressed in a naturally occurring organism. An exemplary artificial DNA sequence is one that contains portions of genes that are ligated together to form an open reading frame that encodes a fusion protein. The portions of DNA that are ligated together can be from a single organism or from more than one organism.

In one embodiment, the composition of the present invention comprises a modified nucleic acid encoding an activator of the invention. For example, in one embodiment, the composition comprises a nucleoside-modified RNA. In one embodiment, the composition comprises a nucleoside-modified mRNA. Nucleoside-modified mRNA have particular advantages over non-modified mRNA, including for example, increased stability, low immunogenicity, and enhanced translation. Nucleoside-modified mRNA useful in the present invention is further described in U.S. Patent No. 8,278,036, which is incorporated by reference herein in its entirety.

Inhibitors

In various embodiments, the composition comprises an inhibitor of a negative regulator of autophagocytosis. In one embodiment, the inhibitor of the invention increases the amount of autophagocytosis. In one embodiment, an inhibitor of the invention decreases the amount of PKCα protein or polypeptide, the amount of mRNA encoding PKCα, the level or activity PKCα, or a combination thereof.

In one embodiment, an inhibitor of the invention decreases the amount or activity of miR-129-2, or a combination thereof. In one embodiment, the inhibitor of the invention comprises an agent that reduces, prevents, or inhibits the interaction of miR-129-2 and PGC-1α.

It will be understood by one skilled in the art, based upon the disclosure provided herein, that a decrease in the level of PKCα protein encompasses the decrease in the expression, including transcription, translation, or both. The skilled artisan will also appreciate, once armed with the teachings of the present invention, that a decrease in the level PKCα includes a decrease in the activity of PKCα. Thus, a decrease in the level or activity of PKCα includes, but is not limited to, decreasing the amount of polypeptide of

PKCα protein, and decreasing transcription, translation, or both, of a nucleic acid encoding PKCα; and it also includes decreasing any activity of PKCα as well.

In one embodiment, the invention provides a generic concept for inhibiting PKCα. In one embodiment, the composition of the invention comprises an inhibitor of PKCα. In one embodiment, the inhibitor is selected from the group consisting of a small interfering RNA (siRNA), shRNA, a microRNA, a guide RNA, a microRNA (miR), an antisense nucleic acid, a ribozyme, an expression vector encoding a transdominant negative mutant, an intracellular antibody, a peptide and a small molecule.

One skilled in the art will appreciate, based on the disclosure provided herein, that one way to decrease the mRNA and/or protein levels of PKCα in a cell is by reducing or inhibiting expression of the nucleic acid encoding PKCα. Thus, the protein level of PKCα in a cell can also be decreased using a molecule or compound that inhibits or reduces gene expression such as, for example, siRNA, shRNA, an antisense molecule or a ribozyme. However, the invention should not be limited to these examples.

In one embodiment, siRNA is used to decrease the level of PKCα. RNA interference (RNAi) is a phenomenon in which the introduction of double-stranded RNA (dsRNA) into a diverse range of organisms and cell types causes degradation of the complementary mRNA. In the cell, long dsRNAs are cleaved into short 21-25 nucleotide small interfering RNAs, or siRNAs, by a ribonuclease known as Dicer. The siRNAs subsequently assemble with protein components into an RNA-induced silencing complex (RISC), unwinding in the process. Activated RISC then binds to complementary transcript by base pairing interactions between the siRNA antisense strand and the mRNA. The bound mRNA is cleaved and sequence specific degradation of mRNA results in gene silencing. See, for example, U.S. Patent No. 6,506,559; Fire et al., 1998, Nature 391(19):306-311; Timmons et al., 1998, Nature 395:854; Montgomery et al., 1998, TIG 14 (7):255-258; David R. Engelke, Ed., RNA Interference (RNAi) Nuts & Bolts of RNAi Technology, DNA Press, Eagleville, PA (2003); and Gregory J. Hannon, Ed., RNAi A Guide to Gene Silencing, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (2003). Soutschek et al. (2004, Nature 432:173-178) describe a chemical modification to siRNAs that aids in intravenous systemic delivery. Optimizing siRNAs involves consideration of overall G/C content, C/T content at the termini, Tm

and the nucleotide content of the 3' overhang. See, for instance, Schwartz et al., 2003, Cell, 115:199-208 and Khvorova et al., 2003, Cell 115:209-216. Therefore, the present invention also includes methods of decreasing levels of PKCα at the protein level using RNAi technology.

In certain embodiments, the modulators described herein comprise short hairpin RNA (shRNA) molecules. shRNA molecules are well known in the art and are directed against the mRNA of a target, thereby decreasing the expression of the target. In certain embodiments, the encoded shRNA is expressed by a cell, and is then processed into siRNA. For example, in certain instances, the cell possesses native enzymes (e.g., dicer) that cleaves the shRNA to form siRNA.

In other related aspects, the invention includes an isolated nucleic acid encoding an inhibitor, wherein an inhibitor such as an siRNA, shRNA or antisense molecule, inhibits PKCα, a derivative thereof, a regulator thereof, or a downstream effector thereof. In one embodiment, an inhibitor such as an siRNA, shRNA or antisense molecule is operably linked to a nucleic acid comprising a promoter/regulatory sequence such that the nucleic acid is preferably capable of directing expression of the iRNA, shRNA or antisense molecule. Thus, the invention encompasses expression vectors and methods for the introduction of exogenous DNA into cells with concomitant expression of the exogenous DNA in the cells such as those described, for example, in Sambrook et al. (2012, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York) and as described elsewhere herein. In another aspect of the invention, PKCα or a regulator thereof, can be inhibited by way of inactivating and/or sequestering PKCα, or a regulator thereof. As such, inhibiting the effects of PKCα can be accomplished by using a transdominant negative mutant.

In another aspect, the invention includes a vector comprising an siRNA, an shRNA or antisense polynucleotide. In one embodiment, the siRNA, shRNA or antisense polynucleotide is capable of inhibiting PKCα. The incorporation of a desired polynucleotide into a vector and the choice of vectors is well-known in the art as described in, for example, Sambrook et al., *supra*.

The siRNA, shRNA or antisense polynucleotide can be cloned into a number of types of vectors as described elsewhere herein. For expression of the siRNA,

shRNA or antisense polynucleotide, at least one module in each promoter functions to position the start site for RNA synthesis.

In order to assess the expression of the siRNA, shRNA or antisense polynucleotide, the expression vector to be introduced into a cell can also contain either a selectable marker gene or a reporter gene or both to facilitate identification and selection of expressing cells from the population of cells sought to be transfected or infected through viral vectors. In other embodiments, the selectable marker may be carried on a separate piece of DNA and used in a co-transfection procedure. Both selectable markers and reporter genes may be flanked with appropriate regulatory sequences to enable expression in the host cells. Useful selectable markers are known in the art and include, for example, antibiotic-resistance genes, such as neomycin resistance and the like.

In one embodiment of the invention, an antisense nucleic acid sequence which is expressed by a plasmid vector is used to inhibit PKCα. The antisense expressing vector is used to transfect a mammalian cell or the mammal itself, thereby causing reduced endogenous expression of PKCα.

Antisense molecules and their use for inhibiting gene expression are well known in the art (*see, e.g.,* Cohen, 1989, In: Oligodeoxyribonucleotides, Antisense Inhibitors of Gene Expression, CRC Press). Antisense nucleic acids are DNA or RNA molecules that are complementary, as that term is defined elsewhere herein, to at least a portion of a specific mRNA molecule (Weintraub, 1990, Scientific American 262:40). In the cell, antisense nucleic acids hybridize to the corresponding mRNA, forming a double-stranded molecule thereby inhibiting the translation of genes.

The use of antisense methods to inhibit the translation of genes is known in the art, and is described, for example, in Marcus-Sakura (1988, Anal. Biochem. 172:289). Such antisense molecules may be provided to the cell via genetic expression using DNA encoding the antisense molecule as taught by Inoue, 1993, U.S. Patent No. 5,190,931.

Alternatively, antisense molecules of the invention may be made synthetically and then provided to the cell. Antisense oligomers of between about 10 to about 30, and more preferably about 15 nucleotides, are preferred, since they are easily synthesized and introduced into a target cell. Synthetic antisense molecules contemplated

by the invention include oligonucleotide derivatives known in the art which have improved biological activity compared to unmodified oligonucleotides (*see* U.S. Patent No. 5,023,243).

Compositions and methods for the synthesis and expression of antisense nucleic acids are as described elsewhere herein.

Ribozymes and their use for inhibiting gene expression are also well known in the art (*see, e.g.,* Cech et al., 1992, J. Biol. Chem. 267:17479-17482; Hampel et al., 1989, Biochemistry 28:4929-4933; Eckstein et al., International Publication No. WO 92/07065; Altman et al., U.S. Patent No. 5,168,053). Ribozymes are RNA molecules possessing the ability to specifically cleave other single-stranded RNA in a manner analogous to DNA restriction endonucleases. Through the modification of nucleotide sequences encoding these RNAs, molecules can be engineered to recognize specific nucleotide sequences in an RNA molecule and cleave it (Cech, 1988, J. Amer. Med. Assn. 260:3030). A major advantage of this approach is the fact that ribozymes are sequence-specific.

There are two basic types of ribozymes, namely, tetrahymena-type (Hasselhoff, 1988, Nature 334:585) and hammerhead-type. Tetrahymena-type ribozymes recognize sequences which are four bases in length, while hammerhead-type ribozymes recognize base sequences 11-18 bases in length. The longer the sequence, the greater the likelihood that the sequence will occur exclusively in the target mRNA species. Consequently, hammerhead-type ribozymes are preferable to tetrahymena-type ribozymes for inactivating specific mRNA species, and 18-base recognition sequences are preferable to shorter recognition sequences which may occur randomly within various unrelated mRNA molecules.

In one embodiment of the invention, a ribozyme is used to inhibit PKCα. Ribozymes useful for inhibiting the expression of a target molecule may be designed by incorporating target sequences into the basic ribozyme structure which are complementary, for example, to the mRNA sequence of PKCα. Ribozymes targeting PKCα may be synthesized using commercially available reagents (Applied Biosystems, Inc., Foster City, CA) or they may be genetically expressed from DNA encoding them.

In one embodiment, the inhibitor of PKCα may comprise one or more components of a CRISPR-Cas system, where a guide RNA (gRNA) targeted to a gene encoding PKCα, and a CRISPR-associated (Cas) peptide form a complex to induce mutations within the targeted gene. In one embodiment, the inhibitor comprises a gRNA or a nucleic acid molecule encoding a gRNA. In one embodiment, the inhibitor comprises a Cas peptide or a nucleic acid molecule encoding a Cas peptide.

When the inhibitor of the invention is a small molecule, a small molecule antagonist may be obtained using standard methods known to the skilled artisan. Such methods include chemical organic synthesis or biological means. Biological means include purification from a biological source, recombinant synthesis and in vitro translation systems, using methods well known in the art.

Combinatorial libraries of molecularly diverse chemical compounds potentially useful in treating a variety of diseases and conditions are well known in the art as are method of making the libraries. The method may use a variety of techniques well-known to the skilled artisan including solid phase synthesis, solution methods, parallel synthesis of single compounds, synthesis of chemical mixtures, rigid core structures, flexible linear sequences, deconvolution strategies, tagging techniques, and generating unbiased molecular landscapes for lead discovery vs. biased structures for lead development.

In a general method for small library synthesis, an activated core molecule is condensed with a number of building blocks, resulting in a combinatorial library of covalently linked, core–building block ensembles. The shape and rigidity of the core determines the orientation of the building blocks in shape space. The libraries can be biased by changing the core, linkage, or building blocks to target a characterized biological structure ("focused libraries") or synthesized with less structural bias using flexible cores.

In other related aspects, the invention includes an isolated peptide inhibitor that inhibits the expression or activity of PKCα. For example, in one embodiment, the peptide inhibitor of the invention inhibits the expression or activity of PKCα directly by binding to PKCα thereby preventing the normal functional activity of PKCα. In another embodiment, the peptide inhibitor of the invention inhibits PKCα by

competing with endogenous PKCα. In yet another embodiment, the peptide inhibitor of the invention inhibits the activity of PKCα by acting as a transdominant negative mutant.

The invention also contemplates an inhibitor of PKCα comprising an antibody, or antibody fragment, specific for PKCα. That is, can inhibit PKCα to provide a beneficial effect. In one embodiment, the antibody specifically binds to PKCα. In one embodiment, the anti-PKCα antibody is a polyclonal antibody. In another embodiment, the anti- PKCα antibody is a monoclonal antibody. In some embodiments, the anti- PKCα antibody is a chimeric antibody. In further embodiments, the anti- PKCα antibody is a humanized antibody. In some embodiments, the antibody is an antibody fragment.

In some embodiments, the antibody is an intact monoclonal or polyclonal antibody, or immunologically portion or active fragment thereof. Thus, in various embodiments, the antibody of invention is a polyclonal antibody, monoclonal antibody, intracellular antibody ("intrabody"), Fv, Fab, Fab', F(ab)2 and F(ab')2, single chain antibody (scFv), heavy chain antibody (e.g., such as a camelid antibody), synthetic antibody, chimeric antibody, or humanized antibodies (see, for example, Harlow et al., 1999, Using Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, NY; Harlow et al., 1989, Antibodies: A Laboratory Manual, Cold Spring Harbor, New York; Houston et al., 1988, Proc. Natl. Acad. Sci. USA 85:5879-5883; Bird et al., 1988, Science 242:423-426). Antibodies can be prepared using intact polypeptides or fragments containing an immunizing antigen of interest. The polypeptide or oligopeptide used to immunize an animal may be obtained from the translation of RNA or synthesized chemically and can be conjugated to a carrier protein, if desired. Suitable carriers that may be chemically coupled to peptides include bovine serum albumin and thyroglobulin, keyhole limpet hemocyanin. The coupled polypeptide may then be used to immunize the animal (e.g., a mouse, a rat, or a rabbit).

As will be understood by one skilled in the art, any antibody that can recognize and bind to an antigen of interest is useful in the present invention. Methods of making and using antibodies are well known in the art. For example, polyclonal antibodies useful in the present invention are generated by immunizing rabbits according to standard immunological techniques well-known in the art (*see*, *e.g.*, Harlow et al., 1988, In: Antibodies, A Laboratory Manual, Cold Spring Harbor, NY). Such techniques

include immunizing an animal with a chimeric protein comprising a portion of another protein such as a maltose binding protein or glutathione (GSH) tag polypeptide portion, and/or a moiety such that the antigenic protein of interest is rendered immunogenic (*e.g.*, an antigen of interest conjugated with keyhole limpet hemocyanin, KLH) and a portion comprising the respective antigenic protein amino acid residues. The chimeric proteins are produced by cloning the appropriate nucleic acids encoding the marker protein into a plasmid vector suitable for this purpose, such as but not limited to, pMAL-2 or pCMX.

However, the invention should not be construed as being limited solely to methods and compositions including these antibodies or to these portions of the antigens. Rather, the invention should be construed to include other antibodies, as that term is defined elsewhere herein, to antigens, or portions thereof. Further, the present invention should be construed to encompass antibodies, *inter alia*, bind to the specific antigens of interest, and they are able to bind the antigen present on Western blots, in solution in enzyme linked immunoassays, in fluorescence activated cells sorting (FACS) assays, in magnetic affinity cell sorting (MACS) assays, and in immunofluorescence microscopy of a cell transiently transfected with a nucleic acid encoding at least a portion of the antigenic protein, for example.

One skilled in the art would appreciate, based upon the disclosure provided herein, that the antibody can specifically bind with any portion of the antigen and the full-length protein can be used to generate antibodies specific therefor. However, the present invention is not limited to using the full-length protein as an immunogen. Rather, the present invention includes using an immunogenic portion of the protein to produce an antibody that specifically binds with a specific antigen. That is, the invention includes immunizing an animal using an immunogenic portion, or antigenic determinant, of the antigen.

Once armed with the sequence of a specific antigen of interest and the detailed analysis localizing the various conserved and non-conserved domains of the protein, the skilled artisan would understand, based upon the disclosure provided herein, how to obtain antibodies specific for the various portions of the antigen using methods well-known in the art or to be developed.

The skilled artisan would appreciate, based upon the disclosure provided herein, that that present invention includes use of a single antibody recognizing a single antigenic epitope but that the invention is not limited to use of a single antibody. Instead, the invention encompasses use of at least one antibody where the antibodies can be directed to the same or different antigenic protein epitopes.

The generation of polyclonal antibodies is accomplished by inoculating the desired animal with the antigen and isolating antibodies which specifically bind the antigen therefrom using standard antibody production methods such as those described in, for example, Harlow et al. (1988, In: Antibodies, A Laboratory Manual, Cold Spring Harbor, NY).

Monoclonal antibodies directed against full length or peptide fragments of a protein or peptide may be prepared using any well-known monoclonal antibody preparation procedures, such as those described, for example, in Harlow et al. (1988, In: Antibodies, A Laboratory Manual, Cold Spring Harbor, NY) and in Tuszynski et al. (1988, Blood, 72:109-115). Quantities of the desired peptide may also be synthesized using chemical synthesis technology. Alternatively, DNA encoding the desired peptide may be cloned and expressed from an appropriate promoter sequence in cells suitable for the generation of large quantities of peptide. Monoclonal antibodies directed against the peptide are generated from mice immunized with the peptide using standard procedures as referenced herein.

Nucleic acid encoding the monoclonal antibody obtained using the procedures described herein may be cloned and sequenced using technology which is available in the art, and is described, for example, in Wright et al. (1992, Critical Rev. Immunol. 12:125-168), and the references cited therein. Further, the antibody of the invention may be "humanized" using the technology described in, for example, Wright et al., and in the references cited therein, and in Gu et al. (1997, Thrombosis and Hematocyst 77:755-759), and other methods of humanizing antibodies well-known in the art or to be developed.

The present invention also includes the use of humanized antibodies specifically reactive with epitopes of an antigen of interest. The humanized antibodies of the invention have a human framework and have one or more complementarity

determining regions (CDRs) from an antibody, typically a mouse antibody, specifically reactive with an antigen of interest. When the antibody used in the invention is humanized, the antibody may be generated as described in Queen, et al. (U.S. Patent No. 6, 180,370), Wright et al., (supra) and in the references cited therein, or in Gu et al. (1997, Thrombosis and Hematocyst 77(4):755-759). The method disclosed in Queen et al. is directed in part toward designing humanized immunoglobulins that are produced by expressing recombinant DNA segments encoding the heavy and light chain complementarity determining regions (CDRs) from a donor immunoglobulin capable of binding to a desired antigen, such as an epitope on an antigen of interest, attached to DNA segments encoding acceptor human framework regions. Generally speaking, the invention in the Queen patent has applicability toward the design of substantially any humanized immunoglobulin. Queen explains that the DNA segments will typically include an expression control DNA sequence operably linked to the humanized immunoglobulin coding sequences, including naturally-associated or heterologous promoter regions. The expression control sequences can be eukaryotic promoter systems in vectors capable of transforming or transfecting eukaryotic host cells or the expression control sequences can be prokaryotic promoter systems in vectors capable of transforming or transfecting prokaryotic host cells. Once the vector has been incorporated into the appropriate host, the host is maintained under conditions suitable for high level expression of the introduced nucleotide sequences and as desired the collection and purification of the humanized light chains, heavy chains, light/heavy chain dimers or intact antibodies, binding fragments or other immunoglobulin forms may follow (Beychok, Cells of Immunoglobulin Synthesis, Academic Press, New York, (1979), which is incorporated herein by reference).

The invention also includes functional equivalents of the antibodies described herein. Functional equivalents have binding characteristics comparable to those of the antibodies, and include, for example, hybridized and single chain antibodies, as well as fragments thereof. Methods of producing such functional equivalents are disclosed in PCT Application WO 93/21319 and PCT Application WO 89/09622.

Functional equivalents include polypeptides with amino acid sequences substantially the same as the amino acid sequence of the variable or hypervariable

regions of the antibodies. "Substantially the same" amino acid sequence is defined herein as a sequence with at least 70%, preferably at least about 80%, more preferably at least about 90%, even more preferably at least about 95%, and most preferably at least 99% homology to another amino acid sequence (or any integer in between 70 and 99), as determined by the FASTA search method in accordance with Pearson and Lipman, 1988 Proc. Nat'l. Acad. Sci. USA 85: 2444-2448. Chimeric or other hybrid antibodies have constant regions derived substantially or exclusively from human antibody constant regions and variable regions derived substantially or exclusively from the sequence of the variable region of a monoclonal antibody from each stable hybridoma.

Single chain antibodies (scFv) or Fv fragments are polypeptides that consist of the variable region of the heavy chain of the antibody linked to the variable region of the light chain, with or without an interconnecting linker. Thus, the Fv comprises an antibody combining site.

Functional equivalents of the antibodies of the invention further include fragments of antibodies that have the same, or substantially the same, binding characteristics to those of the whole antibody. Such fragments may contain one or both Fab fragments or the $F(ab')_2$ fragment. The antibody fragments contain all six complement determining regions of the whole antibody, although fragments containing fewer than all of such regions, such as three, four or five complement determining regions, are also functional. The functional equivalents are members of the IgG immunoglobulin class and subclasses thereof, but may be or may combine with any one of the following immunoglobulin classes: IgM, IgA, IgD, or IgE, and subclasses thereof. Heavy chains of various subclasses, such as the IgG subclasses, are responsible for different effector functions and thus, by choosing the desired heavy chain constant region, hybrid antibodies with desired effector function are produced. Exemplary constant regions are gamma 1 (IgG1), gamma 2 (IgG2), gamma 3 (IgG3), and gamma 4 (IgG4). The light chain constant region can be of the kappa or lambda type.

The immunoglobulins of the present invention can be monovalent, divalent or polyvalent. Monovalent immunoglobulins are dimers (HL) formed of a hybrid heavy chain associated through disulfide bridges with a hybrid light chain. Divalent

immunoglobulins are tetramers (H_2L_2) formed of two dimers associated through at least one disulfide bridge.

Inhibitors of PKCα

In certain embodiments, the invention relates to the use of small molecules for inhibiting PKCα. PKCα antagonists include, but are not limited to, Go6976, Bryostatin 1, Enzastaurin, Staurosporine, Bisindolylmaleimide I, Ro 31-8220 mesylate, Ro 32-0432 hydrochloride, Sotrastaurin, N,N-Dimethyl-D-erythro-sphingosine, PKC412, H 9 dihydrochloride, 10Z-Hymenialdisine, ML-9, HA-156, Bisindolylmaleimide XI hydrochloride, (±)-Palmitoylcarnitine chloride, HBDDE, GF 109203X hydrochloride, HA 100 dihydrochloride, Hypericin, Bisindolylmaleimide X hydrochloride, Bisindolylmaleimide II, Bisindolylmaleimide III, Bisindolylmaleimide IV, NPC-15437, (2S,3R,4E)-2-Azido-3-(tert-butyldimethylsilyl)-erythro-sphingosine, 1-(5-Isoquinolinesulfonyl)-3-methylpiperazine hydrochloride, 1-(5-Isoquinolinesulfonyl)piperazine hydrochloride, 1,2,3,4-Tetrahydrostaurosporine, α-Acetamidocinnamic acid, Bisindolylmaleimide VIII acetate, Cercosporin, Daphnetin, Dequalinium chloride, Hypocrellin A, N-(5-Amino-2-methylphenyl)-4-(3-pyridyl)-2-pyrimidineamine, TMB-8, Verbascoside, Calphostin C, D-erythro-Dihydrosphingosine, PLA2 and PLD inhibitor, D,L-erythro-Dihydrosphingosine, sphingosine kinase inhibitor, L-threo-Dihydrosphingosine and Rottlerin.

Inhibitors of miR-129-2

In one embodiment, the invention relates to compositions comprising modulators (i.e. activators or inhibitors) for use in increasing or decreasing a level of a miR, pre-miR or at least one gene regulated by a miR. As used herein, in general, the terms “activator” and “inhibitor” include but are not limited to a protein, a polypeptide, a peptide, a nucleic acid including, an oligonucleotide or modified oligonucleotide, an antisense oligonucleotide or modified antisense oligonucleotide, cDNA, genomic DNA, an artificial or natural chromosome (e.g. a yeast artificial chromosome) or a part thereof, RNA, including mRNA, tRNA, rRNA or a ribozyme, a peptide nucleic acid (PNA), a nucleotide, a ribonucleotide, a synthetic analog of a nucleotide, a synthetic analog of a

ribonucleotide, a modified nucleotide, a modified ribonucleotide, an amino acid, an amino acid analog, a modified amino acid, a modified amino acid analog, a small molecule, a steroid, a proteoglycan, a lipid, a fatty acid and a carbohydrate. A modulator may be in solution or in suspension (e.g., in crystalline, colloidal or other particulate form). The modulator may be in the form of a monomer, dimer, oligomer, etc, or otherwise in a complex.

In certain embodiments, the composition comprises a modulator that decreases the expression or activity of a miR. Therefore, in one embodiment the composition comprises a miR inhibitor. In one embodiment, a miR inhibitor is a small, chemically modified single-stranded RNA molecules designed to specifically bind to and inhibit endogenous miR molecules (e.g. an anti-miR). In one embodiment, the composition comprises a nucleic acid molecule that encodes a miR inhibitor. In certain embodiments, the miR is miR-129-2.

miRs are small non-coding RNA molecules that are capable of causing post- transcriptional silencing of specific genes in cells by the inhibition of translation or through degradation of the targeted mRNA. A miR can be completely complementary or can have a region of noncomplementarity with a target nucleic acid, consequently resulting in a "bulge" at the region of non-complementarity. A miR can inhibit gene expression by repressing translation, such as when the miR is not completely complementary to the target nucleic acid, or by causing target RNA degradation, which is believed to occur only when the miR binds its target with perfect complementarity. The disclosure also can include double-stranded precursors of miR. A miR can be 18-100 nucleotides in length, and more preferably from 18-80 nucleotides in length. Mature miRs can have a length of 19-30 nucleotides, preferably 21-25 nucleotides, particularly 21, 22, 23, 24, or 25 nucleotides. miR precursors typically have a length of about 70-100 nucleotides and have a hairpin conformation. miRs are generated in vivo from pre- miRs by the enzymes Dicer and Drosha, which specifically process long pre-miR into functional miR. The hairpin or mature microRNAs, or pri- microRNA agents featured in the disclosure can be synthesized in vivo by a cell-based system or in vitro by chemical

synthesis. In one embodiment, a modulator of a miR is a modulator of a miR precursor, e.g. a modulator of a pre-miR.

miR compositions, including, but not limited to, compositions comprising anti-miRs, can be synthesized to include a modification that imparts a desired characteristic. For example, the modification can improve stability, hybridization thermodynamics with a target nucleic acid, targeting to a particular tissue or cell -type, or cell permeability, e.g., by an endocytosis-dependent or -independent mechanism.

Modifications can also increase sequence specificity, and consequently decrease off-site targeting. Methods of synthesis and chemical modifications are described in greater detail below. If desired, miR compositions, including, but not limited to, compositions comprising anti-miRs, may be modified to stabilize the oligonucleiotide molecules against degradation, to enhance half-life, or to otherwise improve efficacy. Desirable modifications are described, for example, in U.S. Patent Publication Nos. 20070213292, 20060287260, 20060035254. 20060008822. and 2005028824, each of which is hereby incorporated by reference in its entirety. For increased nuclease resistance and/or binding affinity to the target, the single- stranded oligonucleotide agents featured in the disclosure can include 2'-O-methyl, 2'-fluorine, 2'-O-methoxyethyl, 2'-O-aminopropyl, 2'-amino, and/or phosphorothioate linkages. Inclusion of locked nucleic acids (LNA), ethylene nucleic acids (ENA), e.g., 2'-4'-ethylene- bridged nucleic acids, and certain nucleotide modifications can also increase binding affinity to the target. The inclusion of pyranose sugars in the oligonucleotide backbone can also decrease endonucleolytic cleavage. A oligonucleotide can be further modified by including a 3' cationic group, or by inverting the nucleoside at the 3'-terminus with a 3 -3' linkage. In another alternative, the 3 '-terminus can be blocked with an aminoalkyl group. Other 3' conjugates can inhibit 3'-5' exonucleolytic cleavage. While not being bound by theory, a 3' may inhibit exonucleolytic cleavage by sterically blocking the exonuclease from binding to the 3' end of the oligonucleotide. Even small alkyl chains, aryl groups, or heterocyclic conjugates or modified sugars (D-ribose, deoxyribose, glucose etc.) can block 3'-5'-exonucleases.

In one embodiment, the anti-miR includes a 2'-modified oligonucleotide containing oligodeoxynucleotide gaps with some or all internucleotide linkages modified

to phosphorothioates for nuclease resistance. The presence of methylphosphonate modifications increases the affinity of the oligonucleotide for its target RNA and thus reduces the IC_5Q. This modification also increases the nuclease resistance of the modified oligonucleotide. It is understood that the methods and reagents of the present disclosure may be used in conjunction with any technologies that may be developed to enhance the stability or efficacy of an inhibitory nucleic acid molecule.

In one embodiment, the miR molecules including, but not limited to, molecules comprising anti-miRs, include nucleotide oligomers containing modified backbones or non-natural internucleoside linkages. Oligomers having modified backbones include those that retain a phosphorus atom in the backbone and those that do not have a phosphorus atom in the backbone. For the purposes of this disclosure, modified oligonucleotides that do not have a phosphorus atom in their internucleoside backbone are also considered to be nucleotide oligomers. Nucleotide oligomers that have modified oligonucleotide backbones include, for example, phosphorothioates, chiral phosphorothioates, phosphorodithioates, phosphotriesters, aminoalkyl-phosphotriesters, methyl and other alkyl phosphonates including 3'-alkylene phosphonates and chiral phosphonates, phosphinates, phosphoramidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriest- ers, and boranophosphates. Various salts, mixed salts and free acid forms are also included. Representative United States patents that teach the preparation of the above phosphorus- containing linkages include, but are not limited to, U.S. Pat. Nos. 3,687,808; 4,469,863; 4,476,301; 5,023,243; 5,177,196; 5,188,897; 5,264,423; 5,276,019; 5,278,302; 5,286,717; 5,321,131; 5,399,676; 5,405,939; 5,453,496; 5,455,233; 5,466,677; 5,476,925; 5,519,126; 5,536,821; 5,541,306; 5,550,111; 5,563,253; 5,571,799; 5,587,361; and 5,625,050, each of which is herein incorporated by reference.

Nucleotide oligomers having modified oligonucleotide backbones that do not include a phosphorus atom therein have backbones that are formed by short chain alkyl or cycloalkyl internucleoside linkages, mixed heteroatom and alkyl or cycloalkyl internucleoside linkages, or one or more short chain heteroatomic or heterocyclic internucleoside linkages. These include those having morpholino linkages (formed in part from the sugar portion of a nucleoside); siloxane backbones; sulfide, sulfoxide and

sulfone backbones; formacetyl and thioformacetyl backbones; methylene formacetyl and thioformacetyl backbones; alkene containing backbones; sulfamate backbones; methyl eneimino and methylenehydrazino backbones; sulfonate and sulfonamide backbones; amide backbones; and others having mixed N, O, S and CH_2 component parts.

Representative United States patents that teach the preparation of the above oligonucleotides include, but are not limited to, U.S. Pat. Nos. 5,034,506; 5,166,315; 5,185,444; 5,214,134; 5,216,141; 5,235,033; 5,264,562; 5,264,564; 5,405,938; 5,434,257; 5,466,677; 5,470,967; 5,489,677; 5,541,307; 5,561 ,225; 5,596,086; 5,602,240; 5,610,289; 5,602,240; 5,608,046; 5,610,289; 5,618,704; 5,623,070; 5,663,312; 5,633,360; 5,677,437; and 5,677,439, each of which is herein incorporated by reference. Nucleotide oligomers may also contain one or more substituted sugar moieties. Such modifications include 2'-O-methyl and 2'- methoxyethoxy modifications. Another desirable modification is 2'-dimethylaminooxyethoxy, 2'-aminopropoxy and 2'-fluoro. Similar modifications may also be made at other positions on an oligonucleotide or other nucleotide oligomer, particularly the 3' position of the sugar on the 3' terminal nucleotide. Nucleotide oligomers may also have sugar mimetics such as cyclobutyl moieties in place of the pentofuranosyl sugar. Representative United States patents that teach the preparation of such modified sugar structures include, but are not limited to, U.S. Pat. Nos. 4,981,957; 5,1 18,800; 5,319,080; 5,359,044; 5,393,878; 5,446,137; 5,466,786; 5,514,785; 5,519,134; 5,567,81 1 ; 5,576,427; 5,591,722; 5,597,909; 5,610,300; 5,627,053; 5,639,873; 5,646,265; 5,658,873; 5,670,633; and 5,700,920, each of which is herein incorporated by reference in its entirety.

In other nucleotide oligomers, both the sugar and the internucleoside linkage, i.e., the backbone, are replaced with groups. Methods for making and using these nucleotide oligomers are described, for example, in "Peptide Nucleic Acids (PNA): Protocols and Applications" Ed. P. E. Nielsen, Horizon Press, Norfolk, United Kingdom, 1999. Representative United States patents that teach the preparation of PNAs include, but are not limited to, U.S. Pat. Nos. 5,539,082; 5,714,331 ; and 5,719,262, each of which is herein incorporated by reference. Further teaching of PNA compounds can be found in Nielsen et al, Science, 1991, 254, 1497- 1500.

In other embodiments, a single stranded modified nucleic acid molecule (e.g., a nucleic acid molecule comprising a phosphorothioate backbone and 2'-OMe sugar modifications is conjugated to cholesterol.

In some examples, the anti-miR composition is at least partially crystalline, uniformly crystalline, and/or anhydrous (e.g., less than 80, 50, 30, 20, or 10% water). In another example, the anti-miR composition is in an aqueous phase, e.g., in a solution that includes water. The aqueous phase or the crystalline compositions can be incorporated into a delivery vehicle, e.g., a liposome (particularly for the aqueous phase), or a particle (e.g., a microparticle as can be appropriate for a crystalline composition). Generally, the miR composition is formulated in a manner that is compatible with the intended method of administration. The anti-miR composition can be formulated in combination with another agent, e.g., another therapeutic agent or an agent that stabilizes an oligonucleotide agent, e.g., a protein that complexes with the oligonucleotide agent. Still other agents include chelators, e.g., EDTA (e.g., to remove divalent cations such as Mg), salts, and RNAse inhibitors (e.g., a broad specificity RNAse inhibitor).

Small Molecule Inhibitors of miRs

Small molecules, including inorganic and organic chemicals, peptides and peptoids, have been reported as small molecule drugs targeting specific miRs (SMIRs). Therefore, in one embodiment, the invention relates to compositions comprising a small molecule inhibitor of miR-129-2. In one embodiment, a small molecule of the invention will have specific binding affinity to a mature miR or a pre-miR.

Anti-miR Oligonucleotides

Anti-miR oligonucleotides (AMOs) are generally single-stranded, chemically modified DNA-like molecules that are designed to be complementary to and inhibit a selected miR. In one embodiment, the composition comprises an AMO targeting miR-129-2. Generally miRs are incorporated into ribonucleoprotein particles (miRNPs) which predominantly act as translational repressors. AMOs are single stranded anti-microRNA molecules which are capable of inhibiting miRNP activity.

In one embodiment, the AMO is a modified oligonucleotides. In one embodiment, the phosphate backbone of the AMO is modified. A modification of an

AMO may include, but is not limited to, a LNA modification, a morpholino modification and a chemical modification. LNA is a bicyclic RNA analogue in which the ribose is locked in a C3'-endo conformation by introduction of a 2'-O,4'-C methylene bridge. Morpholinos are uncharged, inherently resistant to degradation by nucleases. A representative United States patent application that teaches the preparation of such AMOs is published U.S. Application No. 20050182005A1 which is hereby incorporated by reference in its entirety.

In one embodiment, the invention includes a vector for expression of an anti-miR of the invention. In one embodiment, the vector is an expression vector designed to mediate the delivery of small RNAs in mammalian cells. In one embodiment, the expression vector is designed to stably express an anti-miR of the invention. The anti-miR oligonucleotide can be cloned into a number of types of vectors, including but not limited to lentiviral expression vectors.

In order to assess the expression of the anti-miR oligonucleotide, the expression vector to be introduced into a cell can also contain either a selectable marker gene or a reporter gene or both to facilitate identification and selection of expressing cells from the population of cells sought to be transfected or infected through viral vectors. In other embodiments, the selectable marker may be carried on a separate piece of DNA and used in a co-transfection procedure. Both selectable markers and reporter genes may be flanked with appropriate regulatory sequences to enable expression in the host cells. Useful selectable markers are known in the art and include, for example, antibiotic-resistance genes, such as neomycin resistance and the like.

Alternatively, anti-miR oligonucleotides of the invention may be made synthetically and then provided to the cell. Compositions and methods for the synthesis and administration of anti-miR oligonucleotides are as described elsewhere herein.

miR Sponges

In one embodiment, an inhibitor of a miR of the invention may be in the form of a miR sponge. miR sponges are RNA transcripts produced from transgenes expressed in cells that contain multiple binding sites for a target miR. In one embodiment, a miR sponge may be expressed in a cell using an expression vector and administered using

gene therapy methods. In one embodiment, a miR sponge of the invention targets miR-129-2.

Substrates

The present invention provides a scaffold or substrate composition comprising a modulator of the invention, an isolated nucleic acid of the invention, a cell expressing the modulator of the invention, or a combination thereof. For example, in one embodiment, a modulator of the invention, an isolated nucleic acid of the invention, a cell a cell expressing the modulator of the invention, or a combination thereof is incorporated within a scaffold. In another embodiment, a modulator of the invention, an isolated nucleic acid of the invention, a cell expressing the modulator of the invention, or a combination thereof is applied to the surface of a scaffold. The scaffold of the invention may be of any type known in the art. Non-limiting examples of such a scaffold includes a, hydrogel, electrospun scaffold, foam, mesh, sheet, patch, and sponge.

Pharmaceutical Compositions

The formulations of the pharmaceutical compositions described herein may be prepared by any method known or hereafter developed in the art of pharmacology. In general, such preparatory methods include the step of bringing the active ingredient into association with a carrier or one or more other accessory ingredients, and then, if necessary or desirable, shaping or packaging the product into a desired single- or multi-dose unit.

Although the description of pharmaceutical compositions provided herein are principally directed to pharmaceutical compositions which are suitable for ethical administration to humans, it will be understood by the skilled artisan that such compositions are generally suitable for administration to animals of all sorts. Modification of pharmaceutical compositions suitable for administration to humans in order to render the compositions suitable for administration to various animals is well understood, and the ordinarily skilled veterinary pharmacologist can design and perform such modification with merely ordinary, if any, experimentation. Subjects to which

administration of the pharmaceutical compositions of the invention is contemplated include, but are not limited to, humans and other primates, mammals including commercially relevant mammals such as non-human primates, cattle, pigs, horses, sheep, cats, and dogs.

Pharmaceutical compositions that are useful in the methods of the invention may be prepared, packaged, or sold in formulations suitable for ophthalmic, oral, rectal, vaginal, parenteral, topical, pulmonary, intranasal, buccal, or another route of administration. Other contemplated formulations include projected nanoparticles, liposomal preparations, resealed erythrocytes containing the active ingredient, and immunologically-based formulations.

A pharmaceutical composition of the invention may be prepared, packaged, or sold in bulk, as a single unit dose, or as a plurality of single unit doses. As used herein, a "unit dose" is discrete amount of the pharmaceutical composition comprising a predetermined amount of the active ingredient. The amount of the active ingredient is generally equal to the dosage of the active ingredient which would be administered to a subject or a convenient fraction of such a dosage such as, for example, one-half or one-third of such a dosage.

The relative amounts of the active ingredient, the pharmaceutically acceptable carrier, and any additional ingredients in a pharmaceutical composition of the invention will vary, depending upon the identity, size, and condition of the subject treated and further depending upon the route by which the composition is to be administered. By way of example, the composition may comprise between 0.1% and 100% (w/w) active ingredient.

In addition to the active ingredient, a pharmaceutical composition of the invention may further comprise one or more additional pharmaceutically active agents, including, for example, chemotherapeutics, immunosuppressants, corticosteroids, analgesics, and the like.

Controlled- or sustained-release formulations of a pharmaceutical composition of the invention may be made using conventional technology.

As used herein, "parenteral administration" of a pharmaceutical composition includes any route of administration characterized by physical breaching of

a tissue of a subject and administration of the pharmaceutical composition through the breach in the tissue. Parenteral administration thus includes, but is not limited to, administration of a pharmaceutical composition by injection of the composition, by application of the composition through a surgical incision, by application of the composition through a tissue-penetrating non-surgical wound, and the like. In particular, parenteral administration is contemplated to include, but is not limited to, intraocular, intravitreal, subcutaneous, intraperitoneal, intramuscular, intrasternal injection, and kidney dialytic infusion techniques.

Formulations of a pharmaceutical composition suitable for parenteral administration comprise the active ingredient combined with a pharmaceutically acceptable carrier, such as sterile water or sterile isotonic saline. Such formulations may be prepared, packaged, or sold in a form suitable for bolus administration or for continuous administration. Injectable formulations may be prepared, packaged, or sold in unit dosage form, such as in ampules or in multi-dose containers containing a preservative. Formulations for parenteral administration include, but are not limited to, suspensions, solutions, emulsions in oily or aqueous vehicles, pastes, and implantable sustained-release or biodegradable formulations. Such formulations may further comprise one or more additional ingredients including, but not limited to, suspending, stabilizing, or dispersing agents. In one embodiment of a formulation for parenteral administration, the active ingredient is provided in dry (i.e., powder or granular) form for reconstitution with a suitable vehicle (e.g., sterile pyrogen-free water) prior to parenteral administration of the reconstituted composition.

The pharmaceutical compositions may be prepared, packaged, or sold in the form of a sterile injectable aqueous or oily suspension or solution. This suspension or solution may be formulated according to the known art, and may comprise, in addition to the active ingredient, additional ingredients such as the dispersing agents, wetting agents, or suspending agents described herein. Such sterile injectable formulations may be prepared using a non-toxic parenterally-acceptable diluent or solvent, such as water or 1,3-butane diol, for example. Other acceptable diluents and solvents include, but are not limited to, Ringer's solution, isotonic sodium chloride solution, and fixed oils such as synthetic mono- or di-glycerides. Other parentally-administrable formulations which are

useful include those which comprise the active ingredient in microcrystalline form, in a liposomal preparation, or as a component of a biodegradable polymer systems. Compositions for sustained release or implantation may comprise pharmaceutically acceptable polymeric or hydrophobic materials such as an emulsion, an ion exchange resin, a sparingly soluble polymer, or a sparingly soluble salt.

A pharmaceutical composition of the invention may be prepared, packaged, or sold in a formulation suitable for pulmonary administration via the buccal cavity. Such a formulation may comprise dry particles which comprise the active ingredient and which have a diameter in the range from about 0.5 to about 7 nanometers, and preferably from about 1 to about 6 nanometers. Such compositions are conveniently in the form of dry powders for administration using a device comprising a dry powder reservoir to which a stream of propellant may be directed to disperse the powder or using a self-propelling solvent/powder-dispensing container such as a device comprising the active ingredient dissolved or suspended in a low-boiling propellant in a sealed container. Preferably, such powders comprise particles wherein at least 98% of the particles by weight have a diameter greater than 0.5 nanometers and at least 95% of the particles by number have a diameter less than 7 nanometers. More preferably, at least 95% of the particles by weight have a diameter greater than 1 nanometer and at least 90% of the particles by number have a diameter less than 6 nanometers. Dry powder compositions preferably include a solid fine powder diluent such as sugar and are conveniently provided in a unit dose form.

Low boiling propellants generally include liquid propellants having a boiling point of below 65°F at atmospheric pressure. Generally the propellant may constitute 50 to 99.9% (w/w) of the composition, and the active ingredient may constitute 0.1 to 20% (w/w) of the composition. The propellant may further comprise additional ingredients such as a liquid non-ionic or solid anionic surfactant or a solid diluent (preferably having a particle size of the same order as particles comprising the active ingredient).

Formulations of a pharmaceutical composition suitable for parenteral administration comprise the active ingredient combined with a pharmaceutically acceptable carrier, such as sterile water or sterile isotonic saline. Such formulations may

be prepared, packaged, or sold in a form suitable for bolus administration or for continuous administration. Injectable formulations may be prepared, packaged, or sold in unit dosage form, such as in ampules or in multi-dose containers containing a preservative. Formulations for parenteral administration include, but are not limited to, suspensions, solutions, emulsions in oily or aqueous vehicles, pastes, and implantable sustained-release or biodegradable formulations. Such formulations may further comprise one or more additional ingredients including, but not limited to, suspending, stabilizing, or dispersing agents. In one embodiment of a formulation for parenteral administration, the active ingredient is provided in dry (i.e., powder or granular) form for reconstitution with a suitable vehicle (e.g., sterile pyrogen-free water) prior to parenteral administration of the reconstituted composition.

The pharmaceutical compositions may be prepared, packaged, or sold in the form of a sterile injectable aqueous or oily suspension or solution. This suspension or solution may be formulated according to the known art, and may comprise, in addition to the active ingredient, additional ingredients such as the dispersing agents, wetting agents, or suspending agents described herein. Such sterile injectable formulations may be prepared using a non-toxic parenterally-acceptable diluent or solvent, such as water or 1,3-butane diol, for example. Other acceptable diluents and solvents include, but are not limited to, Ringer's solution, isotonic sodium chloride solution, and fixed oils such as synthetic mono- or di-glycerides. Other parentally-administrable formulations that are useful include those that comprise the active ingredient in microcrystalline form, in a liposomal preparation, or as a component of a biodegradable polymer system. Compositions for sustained release or implantation may comprise pharmaceutically acceptable polymeric or hydrophobic materials such as an emulsion, an ion exchange resin, a sparingly soluble polymer, or a sparingly soluble salt.

Additionally, the molecules may be delivered using a sustained-release system, such as semipermeable matrices of solid polymers containing the therapeutic agent. Various forms of sustained-release materials have been established and are well known by those skilled in the art. Sustained-release capsules may, depending on their chemical nature, release the molecules for a few weeks up to over 100 days. Depending

on the chemical nature and the biological stability of the chimeric molecules, additional strategies for molecule stabilization may be employed.

Nucleic acids may be included in any of the above-described formulations as the free acids or bases or as pharmaceutically acceptable salts. Pharmaceutically acceptable salts are those salts that substantially retain the biologic activity of the free bases and which are prepared by reaction with inorganic acids. Pharmaceutical salts tend to be more soluble in aqueous and other protic solvents than are the corresponding free base forms.

In addition to the formulations described previously, the molecules may also be formulated as a depot preparation. Such long acting formulations may be administered by implantation (for example subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the molecules may be formulated with suitable polymeric or hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

Alternatively, other pharmaceutical delivery systems may be employed. Liposomes and emulsions are well-known examples of delivery vehicles that may be used to deliver nucleic acids of the disclosure.

Methods of preventing neural tube defects

In one embodiment, the present invention provides methods for treatment, inhibition, prevention, or reduction of a hyperglycemia induced neural tube defect or a disease or disorder associated with a hyperglycemia induced neural tube defect, by administering a composition comprising an activator of autophagocytosis, as disclosed herein, to a subject in need thereof, optionally in combination with at least one additional agent or therapy. In one embodiment, the method comprises administering a composition comprising an activator of autophagocytosis, as disclosed herein to a subject who is pregnant or seeking to become pregnant for the inhibition, prevention, or reduction of a hyperglycemia induced neural tube defect or a disease or disorder associated with a hyperglycemia induced neural tube defect in an embryo, fetus or infant. In one embodiment, a subject administered the composition of the invention has been diagnosed

as having diabetes, pre-diabetes, or condition associated with a hyperglycemia. In one embodiment, the composition of the invention is administered to a diabetic, prediabetic or hyperglycemic subject who is trying to become pregnant or who is between puberty and menopause and not taking birth control. In one embodiment, the composition of the invention is administered during a preconceptional period, a periconceptional period, a gestational period, or a combination thereof. In one embodiment, a periconceptional period is at least 3 months, at least 2 months, at least one month, at least 4 weeks, at least 3 weeks, at least 2 weeks, at least 1 week, at least 6 days, at least 5 days, at least 4 days, at least 3 days, at least 2 days, or at least 1 day prior to conception. In one embodiment, a gestational period is a period of time between conception and birth. In one embodiment, the composition of the invention is administered daily during days 17 to 30 of gestation.

Therapeutic Methods

Methods of modulating autophagy in a cell comprise contacting cells or subjects with a modulator of autophagy as described herein. The contacting may be by addition of the inhibitor to a fluid surrounding the cells, for example, to the growth media in which the cells are living or existing. The contacting may also be by directly contacting the modulator to the cells. Alternately, the contacting may be by passage of the modulator through a subject, for example, after administration, depending on the route of administration, the modulator may travel through the digestive tract or the blood stream or may be applied or administered directly to cells in need of the autophagy modulation. In one embodiment, the modulator may travel through the umbilical cord or cross the placenta.

In one embodiment, the invention provides methods of modulating autophagy or the level or activity of a regulator thereof, such that the modulation produces a therapeutic effect in a subject, or group of subjects. A therapeutic effect is one that results in an amelioration in the symptoms, or progression of a disease or disorder. In one embodiment, the methods of the invention serve to increase autophagy in an embryo or fetus.

A change in activity can be reflected in terms of the expression of at least one regulator of autophagy. A change in expression can be measured by quantitative or

qualitative measurements of the protein or miR level of a regulator of autophagy, for example by Western blot analysis or rtPCR. The quantitative assay can be used to measure downregulation or upregulation of at least one regulator of autophagy in the presence of a modulator of the invention. In one embodiment, an autophagy activator can be one that down-regulates the level or expression of a negative regulator of autophagy (e.g., PKCα or levels of miR-129-2) by at least about 5 percent compared with a comparator control. In one embodiment, an autophagy activator can be one that up-regulates the level or expression of at least one regulator of mitochondrial function (e.g, PPARγ or PGC-1α) by about at least about 5 percent compared with a comparator control.

The present invention provides a method for treating or preventing a disease or disorder associated with decreased levels of autophagy in a subject in need thereof. It is found herein that decreasing the level of expression or activity of a negative regulator of autophagy or increasing the level of expression or activity of a regulator of mitochondrial function, can increase the level of autophagy, thereby treating the disease or disorder in the subject.

The identified modulators of autophagy may also be combined with other pharmaceutical agents to provide combination therapies. The activator of autophagy may be combined with modulators of one or more of proteasome activity, kinase activity (e.g., receptor tyrosine kinase activity), growth factor pathway activity, or the activity of other cellular pathways. In certain embodiments, an autophagy modulator is used in combination with a therapeutic agent used to treat subjects with a neurodegenerative disease (e.g., acetylcholinesterase inhibitors, neurotransmitter agonists or antagonists). The agents of the combination therapy may be administered in combination or more likely separately. The invention not only provides methods of treating diseases with the inventive combinations but also compositions and kits that include the inventive combination of agents, that is, a modulator of autophagy and another agent.

EXPERIMENTAL EXAMPLES

The invention is further described in detail by reference to the following experimental examples. These examples are provided for purposes of illustration only,

and are not intended to be limiting unless otherwise specified. Thus, the invention should in no way be construed as being limited to the following examples, but rather should be construed to encompass any and all variations which become evident as a result of the teaching provided herein.

Without further description, it is believed that one of ordinary skill in the art can, using the preceding description and the following illustrative examples, make and utilize the present invention and practice the claimed methods. The following working examples therefore are not to be construed as limiting in any way the remainder of the disclosure.

Example 1: Protein kinase Cα suppresses autophagy and induces neural tube defects via miR-129-2 in diabetic pregnancy

Autophagy maintains cellular homeostasis and, thus, promotes cell survival during embryonic development (Fimia et al., 2007, Nature. 447(7148):1121-1125). Autophagy is essential for embryonic development because deletion of the autophagy-related gene 5 (Atg5) results in early embryonic lethality at the four-cell to eight cell stages (Tsukamoto et al., 2008, Science. 321(5885):117-120), and deletion of Beclin1, a key component of the autophagy initiating complex, causes embryonic lethality at E7.5 (Yue et al., 2003, Proc Natl Acad Sci U S A. 100(25):15077-15082). At the whole organism level, two waves of massive autophagy in early lives of mice occur at the time of fertilization and the early neonatal period (Mizushima and Levine, 2010, Nat Cell Biol. 12(9):823-830). At specific tissue levels, autophagy plays an important role for cell differentiation and patterning of many tissues (Mizushima and Levine, 2010, Nat Cell Biol. 12(9):823-830). The developing neuroepithelium has a high level of autophagy during the period of neurulation (Fimia et al., 2007, Nature. 447(7148):1121-1125; Xu et al., 2013, Am J Physiol Endocrinol Metab. 305(5):E667-E678). This high level of autophagy is essential for neurulation or neural tube closure (Fimia et al., 2007, Nature. 447(7148):1121-1125; Xu et al., 2013, Am J Physiol Endocrinol Metab. 305(5):E667-E678). Maternal diabetes suppresses autophagy in neuroepithelial cells leading to NTD formation (Xu et al., 2013, Am J Physiol Endocrinol Metab. 305(5):E667-E678). The present study reveals critical molecular intermediates of a signaling pathway that

mediates the inhibitory effect of maternal diabetes on autophagy in the developing neuroepithelium.

It has been shown that PKC inhibitors trigger autophagy whereas PKC activators suppress starvation- or rapamycin-induced autophagy (Jiang et al., 2010, Biochem Biophys Res Commun. 395(4):471-476). In agreement with the inhibitory effect of PKC on autophagy (Jiang et al., 2010, Biochem Biophys Res Commun. 395(4):471-476), the *in vivo* and *in vitro* evidence presented supports the hypothesis that PKCα is a negative regulator of autophagy and mediates the inhibitory effect of maternal diabetes on autophagy. High basal autophagic activities in neuroepithelial cells are likely required for cell survival because autophagy impairment induces neuroepithelial cell apoptosis in the developing neuroepithelium (Fimia et al., 2007, Nature. 447(7148):1121-1125; Xu et al., 2013, Am J Physiol Endocrinol Metab. 305(5):E667-E678). PKCα-suppressed autophagy under diabetic conditions causes cellular homeostatic imbalance by in favor of pro-apoptotic events. Indeed, *Prkca* gene deletion abolishes pro-apoptotic events including mitochondrial dysfunction, UPR and ER stress. Not surprisingly, removing PKCα ultimately abrogates maternal diabetes-induced neuroepithelial cell apoptosis and consequent NTD formation.

Although PKC directly phosphorylates LC3, its inhibition on autophagy is independent of LC3 phosphorylation (Jiang et al., 2010, Biochem Biophys Res Commun. 395(4):471-476), suggesting that molecular intermediates downstream of PKC may be required for autophagy inhibition induced by PKC. Maternal diabetes-induced autophagy impairment is associated with mitochondrial dysfunction (Xu et al., 2013, Am J Physiol Endocrinol Metab. 305(5):E667-E678). The master regulator of mitochondrial biogenesis and function, PGC-1α, is down-regulated by maternal diabetes. PGC-1α is implicated as a positive regulator of autophagy [32,33]. Using both *in vivo* and *in vitro* approaches, the experiments presented demonstrate, for the first time, unraveled PGC-1α as a downstream effector of PKCα. Further studies reveal that PKCα represses autophagy through PGC-1α-down-regulation. Consistent with the findings in the *Prkca* null embryos, restoring PGC-1α in the neuroepithelium prevents autophagy impairment and ameliorates NTD formation under diabetic conditions. Thus, these findings support a critical role of PGC-1α in embryonic neurulation by stimulating autophagy.

While the information regarding transcriptional regulation of PGC-1α is limited, epigenetic mechanisms are implicated in PGC-1α regulation (Ling et al., 2008, Diabetologia. 51(4):615-622). Because PKCα regulates miRNA expression (Chiang et al., 2010, J Biomed Sci. 17:35) and a recent miRNA profiling study showed altered miRNA profiling in embryos exposed to maternal diabetes (Gu et al., 2015, Toxicol Sci. 144(1):186-196) miRNAs that potentially act downstream of PKCα and repress PGC-1α expression were explored. miR-129-2 is a predicted candidate for the repression of PGC-1α expression. RNA binding assays and functional studies ascertain that PGC-1α is a target gene of miR-129-2, which suppresses PGC-1α expression by degrading its mRNA and inhibiting its protein translation. Maternal diabetes significantly increases miR-129-2 expression. Most importantly, miR-129-2 indeed acts downstream of PKCα. The mechanism underlying PKCα-induced miR-129-2 warrants further investigation. This study reveals a new pathway, PKCα-miR-129-2-PGC-1α, in mediating the teratogenic effect of maternal diabetes by inhibiting autophagy.

The PGC-1 family of transcription coactivators, including PGC-1α, PGC-1β and PRC, regulate mitochondrial function and cell viability (Puigserver and Spiegelman, 2003, Endocr Rev. 24(1):78-90). PGC-1α is abundantly present in the central nervous system (CNS) (Puigserver and Spiegelman, 2003, Endocr Rev. 24(1):78-90). Although *PPARGC1A* gene deletion does not affect embryonic development (Lin et al., 2004, Cell. 119(1):121-35), possibly due to the compensation of PGC-1β, embryos with ubiquitous deletion of the *Prc* gene manifest peri-implantation lethality (He et al., 2012, Dev Dyn. 241(5):975-983) as observed in Atg5 null embryos. However, adult PGC-1α null mice do exhibit CNS dysfunction (Lin et al., 2004, Cell. 119(1):121-35).This evidence suggests a critical role of the PGC-1 family in embryonic neural development, possibly by regulating autophagy. The present study supports the role of PGC-1α in neurulation. PGC-1α overexpression in the neuroepithelium abrogates maternal diabetes-induced autophagy impairment, resolves cellular homeostatic imbalance by preventing mitochondrial dysfunction and ER stress, and ultimately reduces NTD formation. Furthermore, ectopic overexpression of PGC-1α stimulates autophagy. Thus, PGC-1α is an autophagy promoting factor. Without being bound by theory, it is reasoned that PGC-1α-induced autophagy preserves mitochondrial function by removing

maternal diabetes-damaged mitochondria. PGC-1α induces mitochondrial biogenesis and function by increasing gene expression that is essential for mitochondrial proliferation, DNA maintenance, oxidative phosphorylation and ROS detoxification (Puigserver and Spiegelman, 2003, Endocr Rev. 24(1):78-90). Therefore, without being bound by theory, it is possible that PGC-1α overexpression restores cellular homeostasis by directly inducing gene expression essential for mitochondrial function. PGC-1α is a co-activator of the peroxisome proliferator-activated receptor gamma (PPARγ). PPARγ agonists, rosiglitazone and pioglitazone, enhance the action of PGC-1α (Corona and Duchen, 2015, Neurochem Res. 40(2):308-316). Sirtuin activators including resveratrol and SIR1720 can decrease PGC-1α acetylation and, thus, increase PGC-1α activity and its downstream target gene (Lagouge et al., 2006, Cell. 127(6):1109-1122). Therefore PPARγ agonists may be effective in ameliorating diabetic embryopathy.

ER stress and mitochondrial dysfunction are downstream of the PKCα-miR-192-PGC-1α pathway. The experiments presented demonstrate that ER stress is indeed a causal factor in diabetes-induced NTDs (Li, X. et al., 2013, Diabetes, 62:599-608). A recent study used the mitochondrial specific superoxide dismutase 2 to inhibit mitochondrial production of reactive oxygen species and mitochondrial dysfunction leading to amelioration of NTD formation in diabetic pregnancy (Zhong et al., 2016, Free Radic Biol Med. 96:234-244). Thus, ER stress and mitochondrial dysfunction are causally involved in diabetic teratogenesis. All embryos exposed to diabetes exhibit impaired autophagy. A threshold for autophagy impairment may be required for NTD formation. Nevertheless, the level of averaged autophagy activity for all embryos exposed to diabetes is significantly lower than that in all embryos under nondiabetic conditions. Additionally, restoring autophagy activity reduces diabetes-induced NTDs, supporting the causal role of autophagy impairment in diabetic embryopathy. Autophagy gene *Ambra1* deletion leads to massive neuroepithelial cell apoptosis and NTD formation (Fimia, G.M. et al., 2007, Nature, 447:1121-1125). If neuroepithelial cells in the neural fold fusion points undergo apoptosis, the neural fold would fail to be fused (Pai et al., 2012, Birth Defects Res A Clin Mol Teratol. 94(10):817-823). These studies have observed excessive cell apoptosis in the developing neuroepithelium and particularly in

the neural fold fusion points leading to neurulation failure (Yang et al., 2013, Sci Signal. 6(290):ra74).

In summary, this study reveals a mechanism underlying maternal diabetes-suppressed autophagy in the neuroepithelium leading to NTD formation. They demonstrate that PKCα negatively regulates autophagy, whereas PGC-1α promotes autophagy. Altered autophagy may also contribute to the etiology of other defects in diabetic pregnancies. Because autophagy is essential for cardiac morphogenesis (Lee et al., 2014, Autophagy. 10(4):572-587), maternal diabetes-impaired autophagy may contribute to the induction of heart defects. Without being bound by theory, it is hypothesized that autophagy may play an important role in other morphogenetic processes that are affected by maternal diabetes.

The materials and methods employed in these experiments are now described.

Mice

WT C57BL/6J, SOD1-transgenic (SOD1-Tg) (#002298) (Gurney, M.E. et al., 1994, Science, 264: 1772-1775), and PKCα knockout (KO) mice (#009068) (Braz, J.C. et al., 2004, Nature medicine, 10:248-254) were purchased from the Jackson Laboratory (Stock No. 009068, Bar Harbor, Maine) and backcrossed with the C57BL/6J strain for ten generations. Nestin promoter driven PGC-1α transgenic (PGC-1α-Tg) mice in C57BL/6J background were generated. The GFP-LC3 strain was previously reported (Mizushima, N. et al., 2004, Molecular biology of the cell, 15:1101-1111).

Model of maternal diabetes-induced NTDs

A rodent model of Streptozotocin (STZ)-induced diabetes in research of diabetic embryopathy was used (Yang et al., 2013, Sci Signal. 6(290):ra74; Xu, C. et al., 2013, American journal of physiology, 305:E667-678; Li, X. et al., 2013, Diabetes, 62:599-608; Li, X. et al., 2012, Diabetes, 61:2084-2092; Sugimura, Y. et al., 2009, Diabetologia, 52:962-971; Kamimoto, Y. et al., 2010, Diabetologia, 53:2046-2055; Salbaum, J. M. et al., 2010, Birth defects research. Part A, clinical and molecular

teratology, 88:601-611). Briefly, eight- to ten-week old female mice were intravenously injected daily with 75 mg/kg STZ in the tail vein over two days to induce diabetes. Diabetes was defined as 12-hour fasting blood glucose concentrations greater than or equal to 14mM which usually occurred at 3-5 days after STZ injections. No difference was detected in embryonic development between STZ/insulin-treated and non-STZ-treated mice (Yang, P. et al., 2008, American journal of obstetrics and gynecology, 198(130):e131-167), suggesting a lack of residual toxic effect of STZ in the animal model. Insulin pellets (Linshin, Canada) were implanted subcutaneously in diabetic mice to restore euglycemia (glucose concentrations: 4-6 mM) prior to mating (Li, X. et al., 2013, Diabetes, 62:599-608; Li, X. et al., 2012, Diabetes, 61:2084-2092). On day 5.5 of pregnancy (E5.5), insulin pellets were removed to permit frank hyperglycemia (≥ 14mM glucose), so that the developing embryos were exposed to hyperglycemia during neurulation (E8–10.5). Embryos were harvested at E8.75 (2:00 PM at E8.5) for biochemical and molecular analyses. At E10.5, embryos were examined under a Leica MZ16F stereomicroscope (Bannockburn, IL) to identify NTDs in a blinded fashion.

Electron microscopy and GFP-LC3 confocal microscopy

GFP-LC3-Tg mice were used to quantify autophagosome formation *in vivo* (Xu, C. et al., 2013, American journal of physiology, 305: E667-678). GFP florescent images in embryonic neuroepithelial cells were recorded by confocal microscopy using a laser scanning microscope (LSM 510 META, ZEISS) with a plan-apochromat 63X Oil numerical aperture 1.4 objective lens, and excitation wave length for GFP (488 nm) and DAPI (405 nM). All pictures in a given Figure were taken with the same setting. GFP-LC3 punctate foci with a diameter greater than or equal to 20 pixels in each cell were calculated by the Image J software according to the manufacturer's manual. Thus, the images captured the aggregated GFP-LC3 (GFP-LC3 puncta) fluorescent signal that was much stronger than that of individual GFP-LC3 (Mizushima, N. et al., 2004, Molecular biology of the cell, 15:1101-1111). In neuroepithelial cells of DM embryos, individual GFP-LC3 protein was diffused in cytoplasm, didn't form puncta, and had a much lower fluorescent signal that was not captured in the images.

Mitochondrial structures were examined by transmission electron microscopy (EM) in the university's EM core facility. Thick sections (1 μm) were cut and visualized at 100× magnification to identify the neuroepithelia of the E8.75 embryos. Thin sections (80 nm) of identified neuroepithelia were cut and viewed with an electron microscope (Joel JEM-1200EX; Tokyo, Japan) at high resolution (10, 12 and 25 K) to identify the cellular organelle structures.

TUNEL assay

ApopTag Red In Situ Apoptosis Detection Kit (Catalog No: S7165, Millipore) was used to detect apoptosis (Yang et al., 2013, Sci Signal. 6(290):ra74). 10-μm frozen embryonic sections were fixed with 4% PFA in PBS and incubated with TUNEL reaction agents. The percentage of apoptotic cells was obtained by dividing the number of TUNEL positive cells with the total number of cells in a microscopic field and then multiplying by 100 from three separate experiments.

Immunoblotting

30-50 μg protein from one embryo was used and embryos were collected from different dams. Embryos were lysed in a lysis buffer (Cell Signaling, #9803) with a protease inhibitor cocktail (Sigma). Mitochondrial proteins were extracted by the Mitochondria Isolation Kit from Thermo Scientific (#89801). Immunobilon-P or Immunobilon-P^{SQ} (Milllipore) membranes were used for immunoblotting. Membranes were exposed to goat anti-rabbit or goat anti-mouse (Jackson ImmunoResearch Laboratories) or goat anti-rat (Chemicon) secondary antibodies. Signals were detected using SuperSignal West Femto Maximum Sensitivity Substrate kit (Thermo Scientific) and chemiluminescence emitted from the bands was directly captured using the UVP Bioimage EC3 system. Densitometric analyses of chemiluminescence signals were performed using the VisionWorks LS software (UVP).

Transfection and mitochondrion/autophagosome imaging

C17.2 mouse neural stem cells, originally obtained from ECACC (European Collection of Cell Culture), are newborn mouse cerebellar progenitor cells

transformed with retroviral v-myc (Snyder, E.Y. et al., 1992, Cell, 68:33-51; Wang, F. et al., 2015, Diabetes, 64:973-988). HeLa cells stably expressing LC3-GFP (Rothenberg, C. et al., 2010, Human molecular genetics, 19:3219-3232) were provided by the University of Maryland Baltimore. C17.2 cells were transfected with the scramble control siRNA, the PKCα-siRNA (sc-35960, Santa Cruz Biotechnology), the PGC1α-siRNA (sc-38885) or the control siRNA using Lipofectamine RNAiMAX (Invitrogen) according to the manufacture's protocol. There were no mycoplasma contamination in the C17.2 cell line and the LC3-GFP HeLa cell line.

After seeded for 24 hours, LC3-GFP Hela cells were transfected with the PGC-1α vector (Addgene) using Lipofectamine 2000. Cells were collected at different time points with additions of MitoID (Enzo life sciences; Farmingdale, NY, USA) for mitochondria staining and Cyto-ID (the Cyto-ID autophagy detection kit, Cat# ENZ-51031-0050, Enzo life sciences; Farmingdale, NY, USA). The Cyto-ID green dye is specific for selectively staining autophagic vesicles in living cells. According to the manufacturers' instructions, The Cyto-ID autophagy staining, which detects pre-autophagosomes, autophagosomes, and autolysosomes, was validated with known inhibitors and activators of autophagic activity. Briefly, C17.2 cells were incubated in 1X Assay Buffer added with Cyto-ID green dye for 30 minutes at 37°C and protected from light. After washes with 1X Assay Buffer, cells were fixed with 4% PFA in PBS, washed with 1X Assay Buffer and covered with cover slides. Then the stained cells were analyzed by the Nikon Ni-U microscope with a Plan Apo 20× numerical aperture 0.75 objective lens and the Iplab software (Qimaging, V3.95). All pictures in a given Figure were taken with the same setting.

Biotin-labeled miR-129-2 pulldown assay

The biotin-labeled miR-129-2-3p (Dharmacon Lafayette, CO) was transfected into C17.2 cells for 48 hours, and then whole-cell lysates were collected. Cell lysates were mixed with streptavidin-coupled Dynabeads (Invitrogen) and incubated at 4°C on rotator overnight. After the beads were washed thoroughly, the bead-bound RNA was isolated and subjected to RT followed by Real-time PCR analysis. Input RNA was extracted and served as a control.

Determination of the miR-129-2 binding site on PGC-1α mRNA

The full-length PGC1α coding region (CR) or its 3'-UTR and 3'-UTR fragments with the predicted miR-129-2 binding site (BS) or mutated-miR-129-2 binding site (BS-Mut) were amplified and subcloned into the pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega, Madison, WI) to generate the pmirGLO-Luc-PGC1α-CR and pmirGLO-PGC1α-3'UTR and pmirGLO-PGC1α-BS and pmirGLO-PGC1α-BS-Mut. Luciferase activities were measured using the Dual-Luciferase Assay System (Promega), and were normalized by the *Renilla* luciferase activity. Real-time PCR (RT-PCR) and subsequent calculations were performed by the StepOnePlus™ Real-Time PCR System (Applied Biosystem). All primer sequences are listed in Table 1.

Table 1: Sequences of primers. (F: Forward; R: Reverse; CR: coding region for PGC-1α; 3' UTR: 3' UTR for PGC-1α)

Primer name	Primer source	Primer sequence	SEQ ID NO:
GRP94F	Primerbank ID: 6755863a1	TCGTCAGAGCTGATGATGAAGT	1
GRP94R		GCGTTTAACCCATCCAACTGAAT	2
CalnexinF	Primerbank ID: 6671664a1	ATGGAAGGGAAGTGGTTACTGT	3
CalnexinR		GCTTTGTAGGTGACCTTTGGAG	4
eIF2αF	Primerbank ID: 6857781a1	AGTCCCTGCTCGAATCTTCCT	5
eIF2αR		TCCCAAGGCAGAACAGATATACC	6
PDIA3F	Primerbank ID: 6679687a1	CGCCTCCGATGTGTTGGA	7
PDIA3R		CAGTGCAATCCACCTTTGCTAA	8
IRE1αF	Primerbank ID: 13249351a1	ACACCGACCACCGTATCTCA	9
IRE1αR		CTCAGGATAATGGTAGCCATGTC	10
BiPF	Primerbank ID: 31981722a1	ACTTGGGGACCACCTATTCCT	11
BiPR		ATCGCCAATCAGACGCTCC	12
Cox5bF	PrimerBank ID:6753500a1	TTCAAGGTTACTTCGCGGAGT	13
Cox5bR		CGGGACTAGATTAGGGTCTTCC	14

SOD2F	PrimerBank ID:31980762a1	CAGACCTGCCTTACGACTATGG	15
SOD2R		CTCGGTGGCGTTGAGATTGTT	16
TfamF	primerbank: 1575501a1	ATTCCGAAGTGTTTTTCCAGCA	17
TfamR		TCTGAAAGTTTTGCATCTGGGT	18
Nrf1F	primerbank: 13529317a1	TATGGCGGAAGTAATGAAAGAC G	19
Nrf1R		CAACGTAAGCTCTGCCTTGTT	20
CRF		AGCTTTTAAAATGGCTTGGGAC ATGTGCAG	21
CRR		CTAGGTCGACTTACCTGCGCAA GCTTCTC	22
3'UTRF		ATATGCTAGCGGCTGAGGAATG ACAGAGAGA	23
3'UTRR		ACTAGTCGACCTCATGTAACAC CGCGTCTG	24
BS-WTF		CGATGCTAGCTTGGTGACAGTG TGTGTGCG	25
BS-WTR		ATATGTCGACACGGTACCGGAG GCTGAC	26
BS-MutF		AGCACCGACCCCTTCAAATGGC AGCATTTCC	27
BS-MutR		GTTCGTTCTGTTCAGGTGCCCCC AAGTCCT	28
Mmu-miR-129-2 inhibitor	Life technologies: 4464084		
miRNA inhibitor Negative Control	Life technologies: 4464076		
Mmu-miR-129-2 mimic	Life technologies: 4464066		
miRNA Mimic Negative Control	Life technologies: 4464058		
Biotin labeled mmu-miR-129-2	miRBase: MI0000585	AAGCCCUUACCCCAAAAAGCAU	29
Biotin labeled negative control	miRBase: MI0000038	CGCUCAUUCUGCCGGUUGUUAU G	30

Statistical analysis

Sample size was estimated to achieve 80% power based on the previous study (Yang et al., 2013, Sci Signal. 6(290):ra74). Nondiabetic and diabetic dams were

randomly assigned to different experimental groups. Statistical differences were determined by Student's *t*-test for two group comparisons and One-way ANOVA for more than two group comparisons using the SigmaStat 3.5 software. In ANOVA analyses, *Tukey*-tests were used to estimate the significance of the results. Significant difference between groups in NTD incidences was analyzed by the *Chi*-square test. The variance was similar between the groups that were being statistically compared.

The results of the experiments are now described.

Apoptosis and reduced autophagy in diabetes-induced NTDs

Maternal diabetes induced a various type of NTDs including exencephaly, craniorachischisis and spina bifida (Figure 10A). In addition to NTDs, microcephaly was observed (Figure 10A). Exencephaly was the predominant type of NTDs comprising of 74.3% NTDs in the mouse model of diabetic embryopathy (Figure 10A). In an exencephalic embryo, serial histological sectioning revealed that failed neural tube closure occurred in the midbrain and the boundary between the forebrain and hindbrain (Figure 10B, Figure 10C). Accordingly, excessive apoptosis was present in the midbrain and forebrain of exencephalic embryos (Figure 1A, Figure 1B, Figure 1C). In normal embryos from diabetic dams, apoptosis was also present in the midbrain and forebrain, albeit in a less degree compared with that in exencephalic embryos (Figure 1A, Figure 1B, Figure 1C). These findings suggest that apoptosis has to reach a threshold for NTD formation, and neuroepithelial cell apoptosis in normal embryos from diabetic dams may be related to microcephaly. Autophagy represented by LC3-GFP green puncta was diminished in the midbrain, forebrain and hindbrain of exencephalic embryos (Figure 2A, Figure 2B, Figure 2C). There was a slight decrease of autophagy in corresponding brain areas of normal embryos from diabetic dams compared with that of normal embryos from nondiabetic dams (Figure 2A, Figure 2B, Figure 2C). Excessive apoptosis and reduced autophagy are manifested in the brains of NTD embryos, suggesting that these events may be causal events in diabetic embryopathy. Subsequent studies were performed in neurulation stage embryos before neural tube closure to determine the causal relationship between reduced autophagy/ enhanced apoptosis and NTD formation.

Diabetes activates PKCα through oxidative stress

Previous studies demonstrated that phosphorylated (p)-PKCα in whole embryonic lysates from diabetic dams was increased, and that overexpressing superoxide dismutase 1 (SOD1) mitigated oxidative stress and abolished this increase (Li, X. et al., 2011, American journal of obstetrics and gynecology, 205(84):e81-86). Here, where PKCα is activated in the developing embryo in response to hyperglycemia was examined. It was observed that the pro-apoptotic protein kinase Cα (PKCα) was specifically activated, along with enhanced superoxide production, in the developing neuroepithelium by diabetes (Figure 3A, Figure 3B). SOD1 overexpression abrogated diabetes-induced PKCα activation and superoxide production (Figure 3A, Figure 3B).

Prkca gene deletion ameliorates NTDs and restores autophagy

The functionality of PKCα activation in diabetes-induced NTDs was tested using PKCα knockout mice (Braz, J.C. et al., 2004, Nature medicine, 10:248-254). Deletion of the *Prkca* gene ameliorated NTD formation, but did not affect maternal hyperglycemia in diabetic dams (Figure 3C, Figure 3D and Figure 16). *Prkca* deletion prevented maternal diabetes-induced reduction in autophagosome numbers in neuroepithelial cells (Figure 4A). The number of microtubule-associated protein light chain 3 (LC3)-GFP puncta, another index of autophagosome formation, was lower in neuroepithelial cells of embryos exposed to diabetes compared with those in the nondiabetic group (Figure 4B). The reduction of LC3-GFP puncta by diabetes was blocked by *Prkca* deletion (Figure 4B). The decrease in conversion of LC3-I to LC3-II, a biochemical parameter for autophagy, was observed in embryos under diabetic conditions, and this decrease was abrogated in the absence of PKCα (Figure 4C). The down-regulation of autophagy promoting factors, and the up-regulation of autophagy negative regulators, by diabetes were also reversed by *Prkca* deletion (Figure 11A).

To further examine whether PKCα suppresses autophagy, the C17.2 neural stem cell line was utilized (Snyder, E.Y. et al., 1992, Cell, 68:33-51; Wang, F. et al., 2015, Diabetes, 64:973-988), which exhibits high basal autophagic activities as those in neuroepithelial cells of the developing neuroepithelium (Xu, C. et al., 2013, American

journal of physiology, 305:E667-678). Using Cyto-ID staining, it was observed that the constitutively active PKCα (caPKCα) mutant and a PKCα pharmacological activator mimicked the inhibition of autophagy by high glucose (Figure 4D, Figure 4E). In contrast, PKCα siRNA knockdown blunted high glucose-induced autophagy reduction (Figure 4F, Figure 11B, and Figure 11C).

Prkca deletion relieves cellular organelle stress

Because autophagy maintains cellular homeostasis by removing and recycling nutrients from damaged mitochondria and the endoplasmic reticulum (ER) (Mizushima, N. et al., 2010, Nature cell biology, 12:823-830), it was next examined whether *Prkca* deletion-restored autophagy could reduce diabetes-induced defective mitochondria (Xu, C. et al., 2013, Americn journal of physiology, 305: E667-678; Yang, X. et al., 1995, The anatomical record, 241:255-267) and stressed ER (Li, X. et al., 2013, Diabetes, 62: 599-608). Indeed, *Prkca* deletion reduced the number of defective mitochondria in neuroepithelial cells of embryos under maternal diabetic conditions (Figure 5A, Figure 11D). Maternal diabetes-induced mitochondrial translocation of the pro-apoptotic Bcl-2 family members, Bak, Bax, Puma and Bim, was inhibited by *Prkca* deletion (Figure 5B, Figure 5C, Figure 5D, Figure 5E). Other mitochondrial dysfunction markers, phosphorylation of Bad and cleaved Bid (tBid) under diabetic conditions, were also diminished by *Prkca* deletion (Figure 11E, Figure 11F). These results support the hypothesis that maternal diabetes-induced mitochondrial dysfunction is prevented by deleting the *Prkca* gene.

The major function of the ER is to post-translationally modify and properly fold the newly-synthesized proteins into dimensional structures. Accumulation of misfolded proteins triggers the unfolded protein response (UPR) and ER stress (Ron, D. et al., 2007, Molecular cell biology, 8:519-529). The activation of the major UPR sensors, IRE1α and PERK, mediates the pro-apoptotic effect of ER stress (Ron, D. et al., 2007, Molecular cell biology, 8:519-529). ER stress is a causal event in the induction of diabetic embryopathy because inhibition of ER stress by 4-phenylbutyric acid blocks high glucose-induced NTD formation (Li, X. et al., 2013, Diabetes, 62:599-608). Autophagy can resolve ER stress (Xu, C. et al., 2013, American journal of Physiology,

305:E667-678; Bachar-Wikstrom, E. et al., 2013, Diabetes, 62:1227-1237). Because *Prkca* deletion reversed diabetes-repressed autophagy, next it was sought to determine whether *Prkca* deletion inhibits diabetes-induced ER stress. Maternal diabetes-induced phosphorylation of IRE1α and PERK, and their downstream effectors, CHOP up-regulation and eIF2α phosphorylation, X-box binding protein 1 (XBP1) mRNA cleavage and up-regulation of ER chaperone genes were abrogated by *Prkca* deletion (Figure 6A, Figure 6B, and Figure 12).

Excess apoptosis in embryonic neuroepithelial cells accounts for NTD induction in diabetic embryopathy (Yang et al., 2013, Sci Signal. 6(290):ra74; Xu, C. et al., 2013, American journal of Physiology, 305:E667-678; Li, X. et al., 2013, Diabetes, 62:599-608; Li, X. et al., 2012, Diabetes, 61:2084-2092; Wang, F. et al., 2015, American journal of obstetrics and gynecology, 213:125-134; Wang, F. et al., 2015, American journal of obstetrics and gynecology, 212(5):650-e1; Wu, Y. et al., 2015, American journal of obstetrics and gynecology, 212(6):802-e1). Because *Prkca* deletion inhibited the two pro-apoptotic responses, mitochondrial dysfunction and ER stress, it was hypothesized that deleting *Prkca* would block neuroepithelial cell apoptosis. To test this hypothesis, caspase cleavage and the percentage of apoptotic cells were measured. Maternal diabetes-induced caspase 3, 8 and neuroepithelial cell apoptosis were diminished by *Prkca* deletion (Figure 6C, Figure 6D, Figure 6E).

PKCα up-regulates miR-129-2 that silences PGC-1αexpression

Next, it was looked into how PKCα mediates the adverse effects of maternal diabetes leading to autophagy impairment and cellular organelle stress. PGC-1α a member of the peroxisome proliferator-activated receptor c coactivator 1 (PGC1) family of transcriptional coactivators, is a positive regulator of mitochondrial biogenesis and function, and plays an important role in many metabolic processes (Austin, S. et al., 2012, Journal of cell science, 125:4963-4971). PGC-1α is also implicated in autophagy induction by exercise and fiber type conversion in muscle (Lira, V. et al., 2013, FASEB journal: official publication of the Federation of American Societies for Experimental Biology, 27:4184-4193; Takikita, S. et al., 2010, Plos one, 5:e15239). It was found that both PGC-1α mRNA and protein abundance were significantly down-regulated by

maternal diabetes, and were restored upon the removal of the *Prkca* gene (Figure 7A, Figure 7B). Silencing PKCα by siRNA reverted high glucose-suppressed PGC-1α expression (Figure 7C), whereas the caPKCα mutant simulated the inhibitory effect of high glucose on PGC-1α expression in C17.2 cells (Figure 7D). These results support the hypothesis that PKCα mediates the inhibitory effect of high glucose on PGC-1α expression.

To investigate the mechanism whereby PKCα suppresses PGC-1α expression, it was focused on miRNAs because it has been demonstrated that PKCα regulates miRNA expression (Chiang, C. et al., 2010, Journal of biomedical science, 17:35). Additionally, there is a drastic miRNA profiling change during mouse neurulation (Mukhopadhyay, P. et al., 2011, Birth defects research. Part A, Clinical and molecular teratology, 91:744-762), and miRNAs are implicated in human NTD formation (Gu, H. et al., 2012, Journal of neurochemistry, 122:641-649). microRNAs are non-coding small RNAs that repress gene expression by either degrading target mRNAs or blocking translation or both (Lee, R.C. et al., 1993, Cell, 75:843-854). A miRNA target prediction algorithm (www.microrna.org) revealed miR-129-2 as a potential negative regulator of PGC-1α expression, suggesting a role of miR-129-2 in diabetic embryopathy. To determine whether miR-129-2 targets PGC-1α mRNA, an RNA pull-down assay was performed using biotin-labeled miR-129-2. PGC-1α mRNA was enriched about 7-fold in biotin-labeled miR-129-2 (Figure 13A). The miR-129-2 mimic repressed the luciferase reporter activities driven by the 3'-untranslated region (UTR) but not the coding region or the binding site mutated 3'-UTR of PGC-1α mRNA (Figure 13B).

The miR-129-2 mimic mimicked high glucose to down-regulate PGC-1α in cultured C17.2 cells (Figure 7E, Figure 7F, and Figure 13C, Figure 13D). The miR-129-2 inhibitor, which forms a duplex with endogenous miR-129-2 and inactivates it, slightly increased PGC-1α expression at 50 nM but reduced PGC-1α expression at high concentrations, probably due to cell toxicity (Figure 13E, Figure 13F). Under normal glucose condition, PGC-1α expression in cultured C17.2 cells reached a plateau that could not be further increased by the miR-129-2 inhibitor (Figure 13E, Figure 13F). Nevertheless, 50 nM miR-129-2 inhibitor abolished high glucose-inhibited PGC-1α protein expression and partially restored PGC-1α mRNA expression in cultured C17.2

cells (Figure 7G, Figure 7H). Thus, miR-129-2 represses PGC-1α expression by degrading mRNA and inhibiting translation. An RNA-immunoprecipitation (RIP) assay was used to assess whether PGC-1α mRNA and miR-129-2 are co-enriched in the RNA-induced silencing complex (RISC). Maternal diabetes enhanced the co-presence of PGC-1α miRNA and miR-129-2 in RISC (Figure 7I, Figure 7J), suggesting that PGC-1α is a miR-129-2 target *in vivo*. Both *in vitro* and *in vivo* data support the hypothesis that maternal diabetes- or high glucose *in vitro*-induced miR-129-2 represses PGC-1α expression.

miR-129-2 is downstream of PKCα because caPKCα simulated high glucose in up-regulating miR-129-2 (Figure 7K, and Figure 13G), and siRNA knockdown of PKCα, or *Prkca* gene deletion blocked high glucose- or diabetes-increased miR-129-2 expression, respectively (Figure 7L, Figure 7M).

PGC-1α overexpression reverses diabetes-inhibited autophagy

To explore the functional consequence of PGC-1α up-regulation, a transgenic mouse line was generated in which *PPARGC1A* gene, along with GFP expression, were driven by the promoter of nestin, a neural stem cell marker (Figure 8A). Consistent with prior reports (Yang et al., 2013, Sci Signal. 6(290):ra74; Petersen, PH. et al., 2002, Nature, 419:929-934; Zimmerman, L. et al., 1994, Neuron, 12:11-24) that the nestin promoter-driven transgene expression occurs at E8.0 onward, GFP signals were robustly present in the E8.5 neuroepithelia of *PPARGC1A* transgene positive (*PPARGC1A*$^+$) embryos but not wild-type (WT) embryos (Figure 8A). Maternal diabetes-reduced autophagosome formation was restored in neuroepithelial cells of *PPARGC1A*$^+$ embryos, which had a comparable number of autophagosomes to that of neuroepithelial cells of WT embryos under nondiabetic conditions (Figure 8B). The reduction in converting LC3-I to LC3-II by diabetes, was abrogated in *PPARGC1A*$^+$ embryos (Figure 8C). Diminished LC3-GFP puncta in neuroepithelial cells under diabetic conditions was reverted by the *PPARGC1A* transgene expression (Figure 8D). Additionally, maternal diabetes-altered autophagy-related gene expression was corrected in *PPARGC1A*$^+$ embryos (Figure 14A).

PGC-1α directly activates autophagy

To confirm the autophagy-promoting effect of PGC-1α, the LC3-GFP autophagy reporter cell line was used (Rothenberg, C. et al., 2010, Human molecular genetics, 19:3219-3232). PGC-1α transfected cells displayed robust LC3-GFP puncta, autophagosomes (Figure 14B). In a time-course experiment, LC3-GFP puncta as well as mitochondrial mass were induced as early as only 6 hours after ectopic PGC-1α overexpression (Figure 8E), suggesting that autophagy induction by PGC-1α overexpression is not a secondary effect of mitochondrial biogenesis. Knocking down PGC-1α using siRNA significantly attenuated autophagosome formation (Figure 8F, and Figure 14C, Figure 14D), whereas ectopic PGC-1α overexpression inhibited high glucose-suppressed autophagy (Figure 14E). Therefore, PGC-1α is an autophagy promoting factor.

PGC-1α overexpression prevents diabetes-induced NTDs

Autophagy is essensial for neural tube closure (Fimia, G.M. et al., 2007, Nature, 447:1121-1125; Xu, C. et al., 2013, American journal of physiology, 305:E667-678), and *PPARGC1A* overexpression restores autophagy that is suppressed by diabetes. To test the effect of *PPARGC1A* overexpression on NTD formation in diabetic pregnancies, *PPARGC1A* transgenic males mated with nondiabetic or diabetic WT females to produce WT and *PPARGC1A*$^{+}$ embryos under the same maternal environment. Under nondiabetic conditions, *PPARGC1A* overexpression did not affect blood glucose levels and NTD rate (Figure 8G, Figure 8H and Figure 17). Only 2.2% of *PPARGC1A*$^{+}$ embryos under diabetic conditions exhibited NTDs, which was significantly lower compared to the 22.7% NTD rate of their WT littermates under diabetic conditions (Figure 8H and Figure 17). The NTD rate in *PPARGC1A*$^{+}$ embryos from diabetic dams was comparable to those of WT and *PPARGC1A*$^{+}$ embryos from nondiabetic parents (Figure 8H and Figure 17). Thus, similar to the findings in *Prkca* gene deletion settings, *PPARGC1A* overexpression ameliorates diabetes-induced NTD formation.

To delineate the mechanism underlying the beneficial effect of *PPARGC1A* overexpression on NTD prevention, indices of mitochondrial dysfunction,

ER stress and apoptosis were assessed. Diabetes-increased number of defective mitochondria and diabetes-repressed mitochondrial gene expression were reverted by *PPARGC1A* overexpression (Figure 9A, Figure 9B). Diabetes-increased p-PERK, p-eIF2α, p-IRE1α and CHOP abundance, ER chaperone gene expression and XBP1 cleavage were abrogated in *PPARGC1A*$^{+}$ embryos (Figure 9C, Figure 15A, Figure 15B, Figure 15C). *PPARGC1A* overexpression blocked diabetes-induced caspase 3 and 8 cleavage, and neuroepithelial cell apoptosis (Figure 9D, Figure 9E, Figure 9F).

Taken together, maternal diabetes-induced PKCα activation increased miR-129-2 expression, which repressed PGC-1α expression by degrading its mRNA and inhibiting translation (Figure 9G). Either *Prkca* deletion or *PPARGC1A* overexpression restored diabetes-suppressed autophagy and cellular homeostasis, and thus prevented NTD formation (Figure 9G). These findings unravel a potential new kinase pathway in autophagy regulation and provide the mechanistic basis for targeting PKCα, miR-129-2, and PGC-1α for intervention for maternal diabetes-induced NTDs (Figure 9G).

CLAIMS

What is claimed is:

1. A method for treating or preventing a hyperglycemia-induced neural tube defect or a disease or disorder associated with hyperglycemia-induced neural tube defects, comprising administering to a subject a composition comprising an agonist of autophagy.

2. The method of claim 1, wherein the agonist of autophagy is selected from the group consisting of an activator of PGC-1α, an activator of PPAR-γ, and an activator of Sirtuin-1.

3. The method of claim 2, wherein the activator is selected from the group consisting of a protein, a peptide, a peptidomimetic, an antibody, a ribozyme, an organic compound, an inorganic compound, a small molecule, a nucleic acid, a vector, and an antisense nucleic acid molecule.

4. The method of claim 3, wherein the activator of PGC-1α is selected from the group consisting of ZLN005, telmisartan and fenofibrate.

5. The method of claim 3, wherein the activator of PPAR-γ is selected from the group consisting of thiazolidinediones, rosiglitazone, pioglitazone, honokiol, amorfrutin 1, amorfrutin B, amorphastilbol, roscovitine, aleglitazar, muraglitazar, saroglitazar and tesaglitazar.

6. The method of claim 3, wherein the activator of Sirtuin-1 is selected from the group consisting of Resveratrol, resVida, Lonevinex, SRT501, Pterostilbene, SRT1720, SRT2104, SRT2379, berberine, acetylsalicylic acid, Metformin, AICAR, AZD-769662 oxaloacetate, and rapamycin.

7. The method of claim 1, wherein the agonist of autophagy is selected from the group consisting of 5,6- epoxyeicosatrienoic acid (EET), 8,9-EET, 11,12-EET, 14,15-EET, epoxyeicosatrienoic acid analogue EET-A, and (±)14(15)-EET.

8. The method of claim 1, wherein the agonist of autophagy is an inhibitor of PKCα.

9. The method of claim 8, wherein the inhibitor is selected from the group consisting of a protein, a peptide, a peptidomimetic, an antibody, a ribozyme, an organic compound, an inorganic compound, a small molecule, a nucleic acid, a vector, and an antisense nucleic acid molecule.

10. The method of claim 9, wherein the inhibitor of PKCα is selected from the group consisting of Go6976, Bryostatin 1, Enzastaurin, Staurosporine, Bisindolylmaleimide I, Ro 31-8220 mesylate, Ro 32-0432 hydrochloride, Sotrastaurin, N,N-Dimethyl-D-erythro-sphingosine, PKC412, H 9 dihydrochloride, 10Z-Hymenialdisine, ML-9, HA-156, Bisindolylmaleimide XI hydrochloride, (±)-Palmitoylcarnitine chloride, HBDDE, GF 109203X hydrochloride, HA 100 dihydrochloride, Hypericin, Bisindolylmaleimide X hydrochloride, Bisindolylmaleimide II, Bisindolylmaleimide III, Bisindolylmaleimide IV, NPC-15437, (2S,3R,4E)-2-Azido-3-(tert-butyldimethylsilyl)-erythro-sphingosine, 1-(5-Isoquinolinesulfonyl)-3-methylpiperazine hydrochloride, 1-(5-Isoquinolinesulfonyl)piperazine hydrochloride, 1,2,3,4-Tetrahydrostaurosporine, α-Acetamidocinnamic acid, Bisindolylmaleimide VIII acetate, Cercosporin, Daphnetin, Dequalinium chloride, Hypocrellin A, N-(5-Amino-2-methylphenyl)-4-(3-pyridyl)-2-pyrimidineamine, TMB-8, Verbascoside, Calphostin C, D-erythro-Dihydrosphingosine, PLA2 and PLD inhibitor, D,L-erythro-Dihydrosphingosine, sphingosine kinase inhibitor, L-threo-Dihydrosphingosine and Rottlerin.

11. The method of claim 1, wherein the agonist of autophagy is an inhibitor of miR-129-2.

12. The method of claim 11, wherein the inhibitor is selected from the group consisting of a small molecule, an anti-miR oligonucleotide (AMO), and a miR sponge.

13. The method of claim 1, wherein the subject has been diagnosed as having a condition associated with hyperglycemia.

14. The method of claim 13, wherein the subject is diabetic.

15. The method of claim 14, wherein the subject is pregnant or trying to conceive.

16. The method of claim 15, wherein the method prevents or reduces the occurrence of a hyperglycemica-induced neural tube defect in an embryo or fetus.

17. The method of claim 1, wherein the disease or disorder associated with hyperglycemia-induced neural tube defects is selected from the group consisting of: congenital heart defect, congenital heart disease, spina bifida, exencephaly, craniorachischisis, microcephaly, chiari malformation, anencephaly and fetal or infant death or a combination thereof.

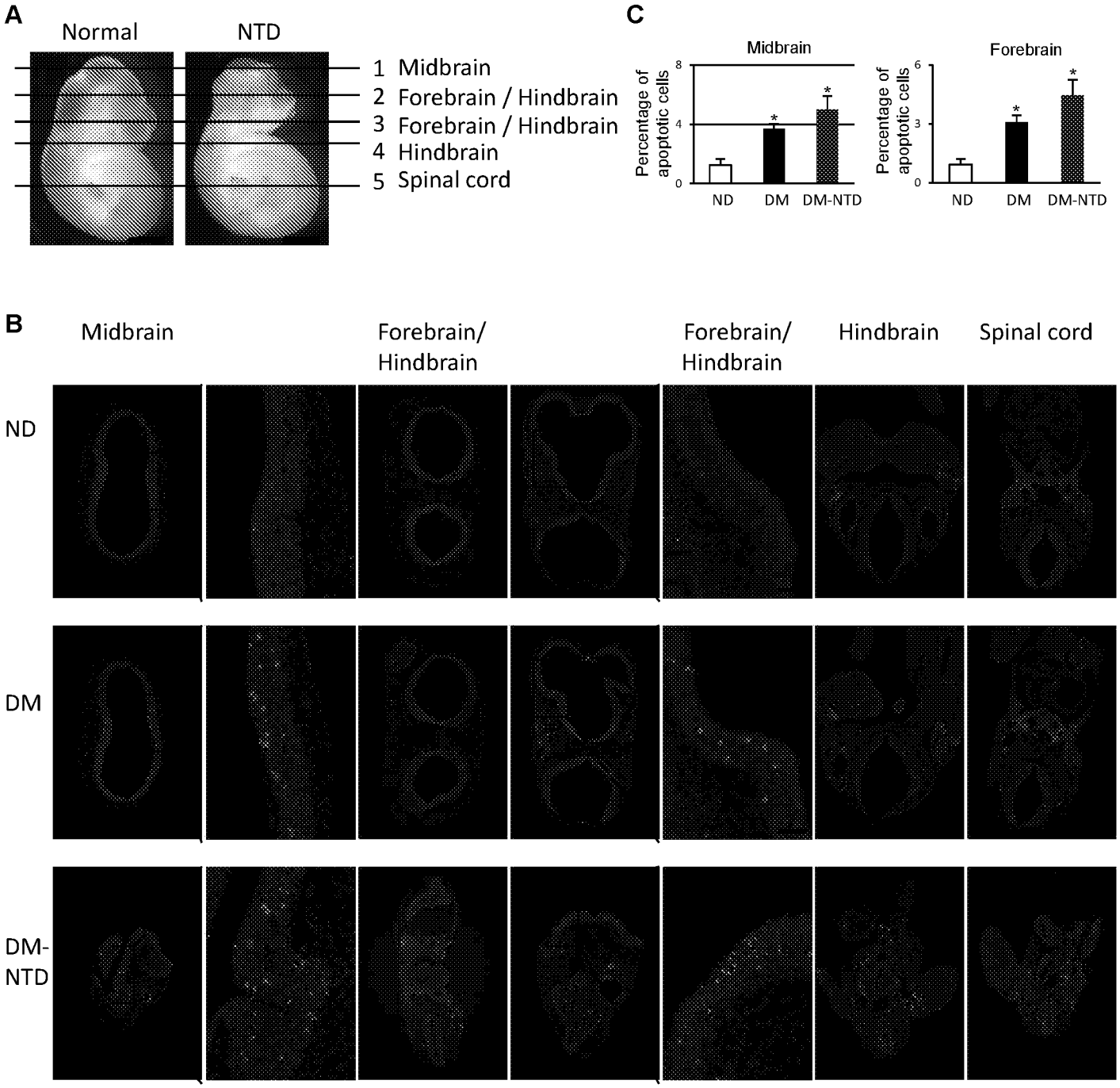

A
Normal
NTD
1 Midbrain
2 Forebrain / Hindbrain
3 Forebrain / Hindbrain
4 Hindbrain
5 Spinal cord
C
Midbrain
Percentage of apoptotic cells
8
4
0
ND
DM
DM-NTD
Forebrain
Percentage of apoptotic cells
6
3
0
ND
DM
DM-NTD
B
Midbrain
Forebrain/ Hindbrain
Forebrain/ Hindbrain
Hindbrain
Spinal cord
ND
DM
DM-NTD

Fig. 1A – 1C

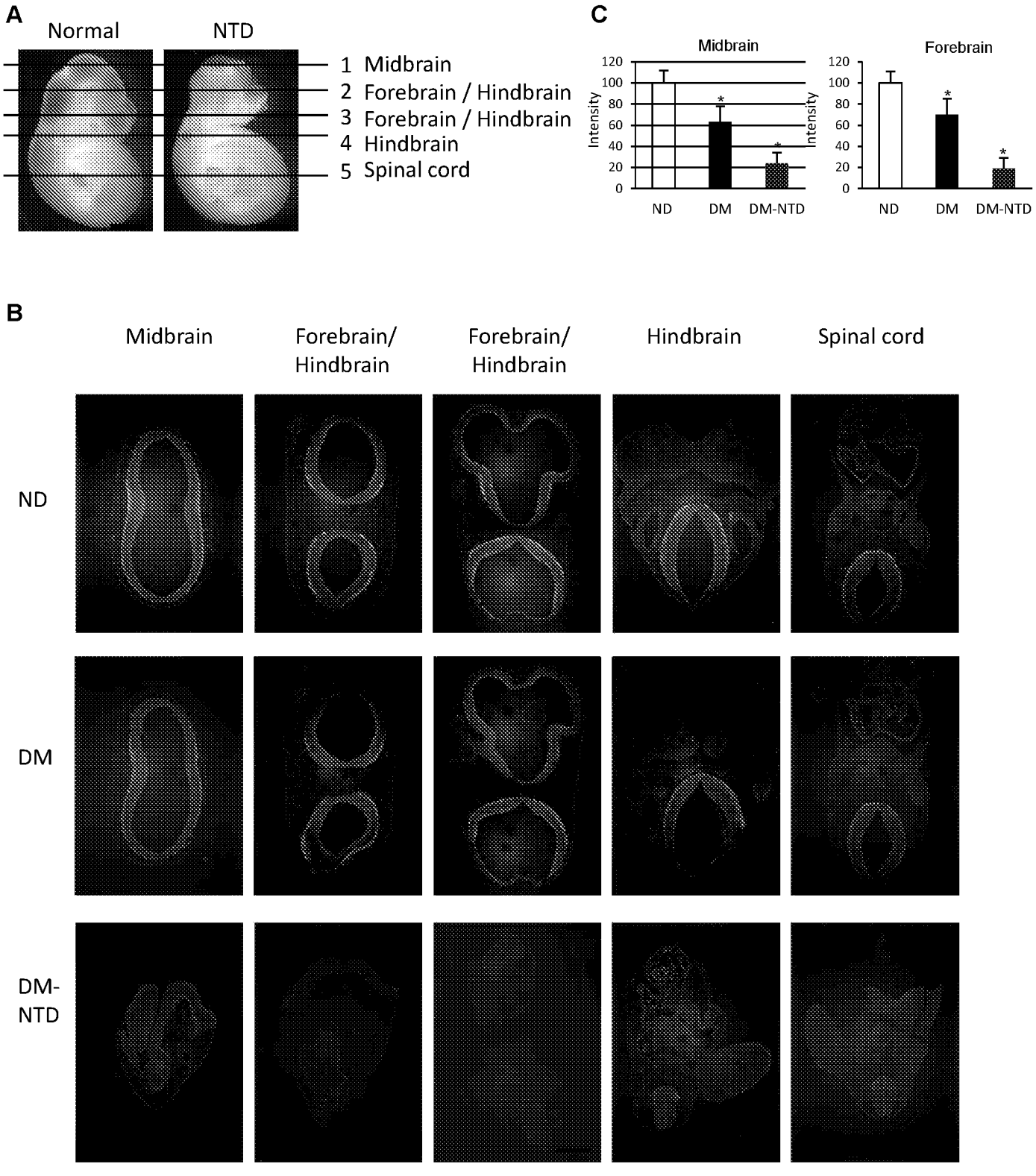

A
Normal
NTD
1 Midbrain
2 Forebrain / Hindbrain
3 Forebrain / Hindbrain
4 Hindbrain
5 Spinal cord
C
Midbrain
Forebrain
Intensity
120
100
80
60
40
20
0
ND
DM
DM-NTD
B
Midbrain
Forebrain/ Hindbrain
Forebrain/ Hindbrain
Hindbrain
Spinal cord
ND
DM
DM-NTD

Fig. 2A – 2C

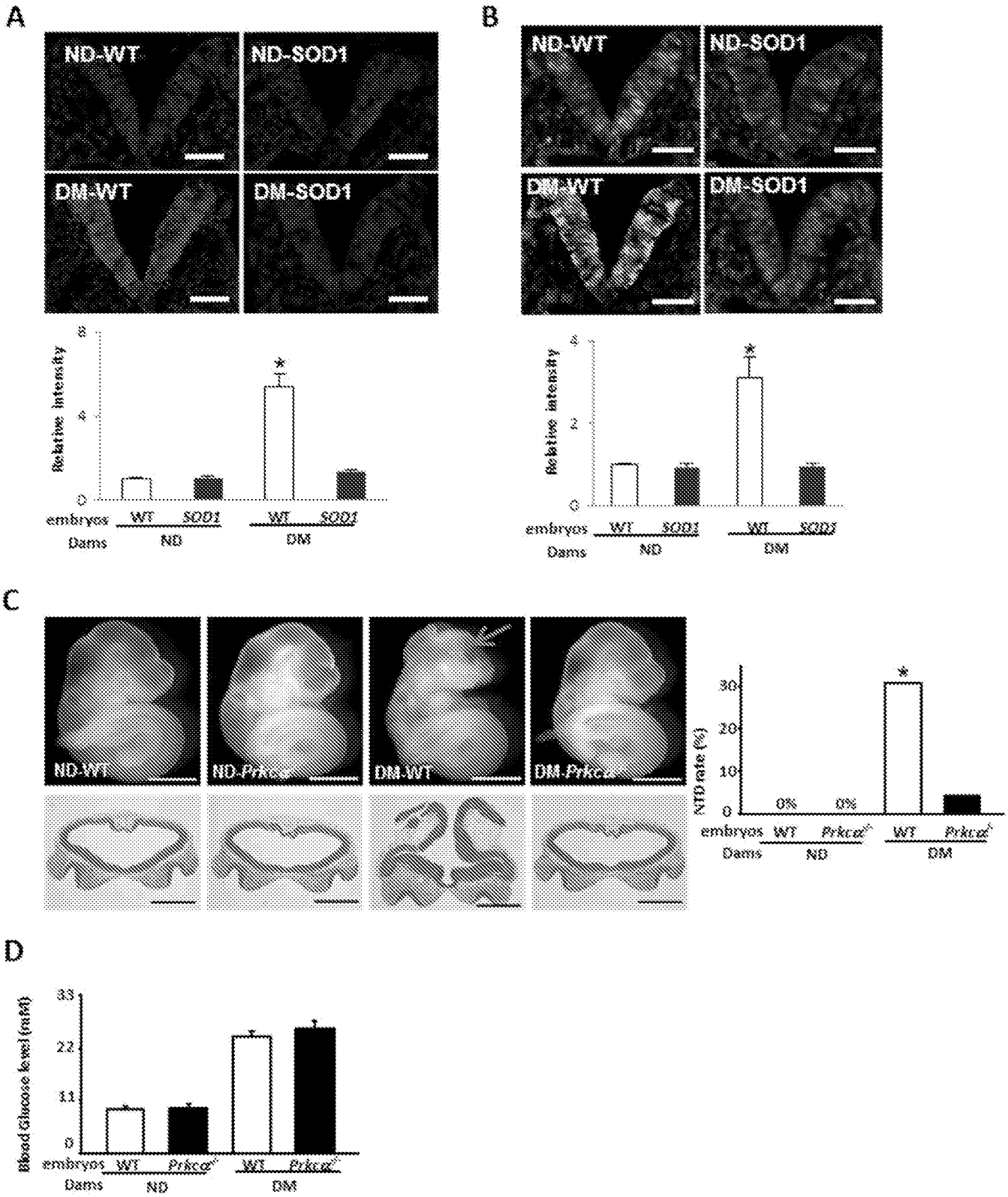

A
ND-WT
ND-SOD1
DM-WT
DM-SOD1
Relative intensity
embryos WT SOD1 WT SOD1
Dams ND DM
B
ND-WT
ND-SOD1
DM-WT
DM-SOD1
Relative intensity
embryos WT SOD1 WT SOD1
Dams ND DM
C
ND-WT
ND-Prkcα-/-
DM-WT
DM-Prkcα-/-
NTD rate (%)
0% 0%
embryos WT Prkcα-/- WT Prkcα-/-
Dams ND DM
D
Blood Glucose level (mM)
embryos WT Prkcα-/- WT Prkcα-/-
Dams ND DM

Fig. 3A – 3D

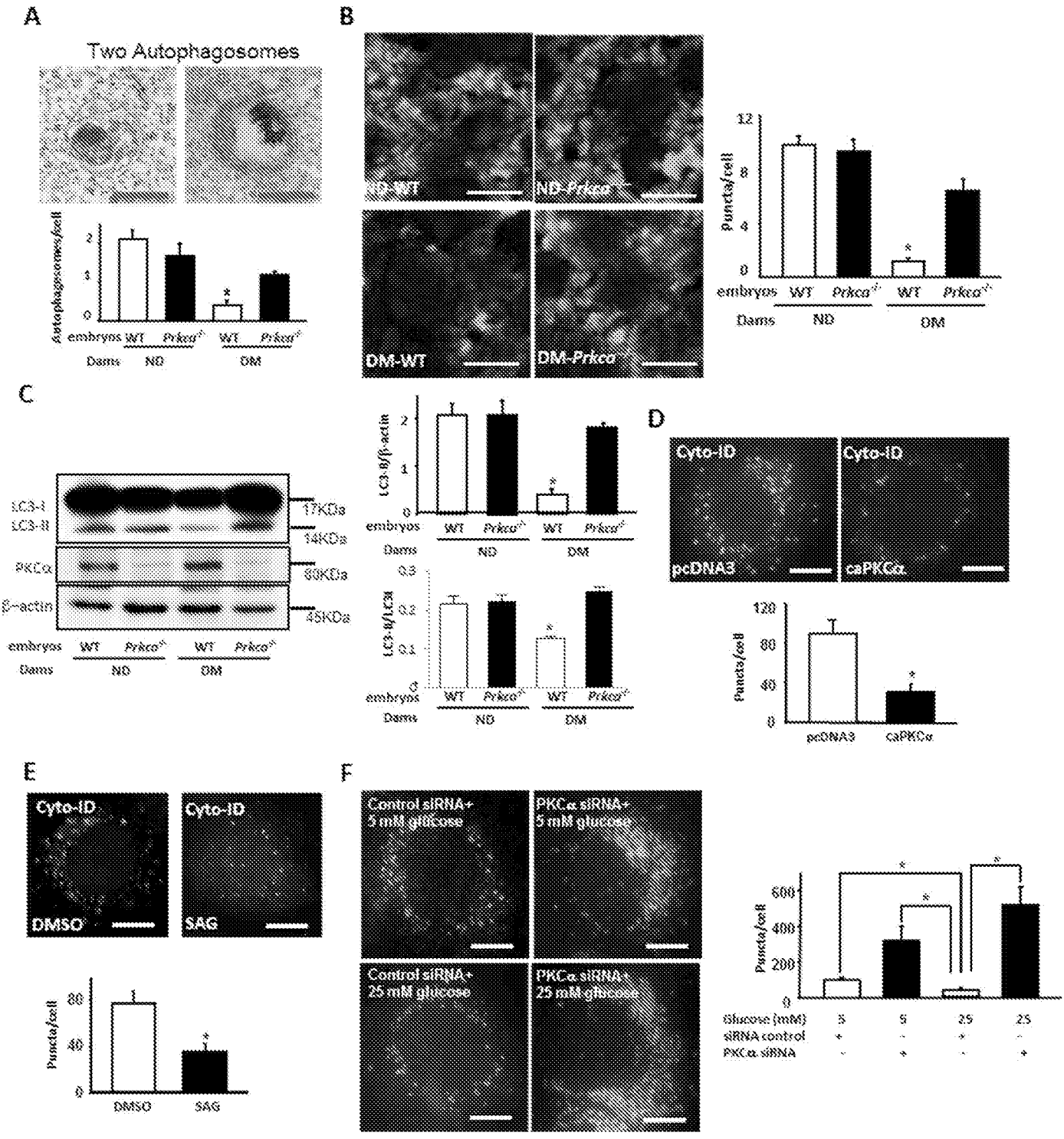

A
Two Autophagosomes
Autophagosomes/cell
embryos WT Prkca-/- WT Prkca-/-
Dams ND DM
B
ND-WT
ND-Prkca-/-
DM-WT
DM-Prkca-/-
Puncta/cell
embryos WT Prkca-/- WT Prkca-/-
Dams ND DM
C
LC3-I
LC3-II
17KDa
14KDa
PKCα
80KDa
β-actin
45KDa
embryos WT Prkca-/- WT Prkca-/-
Dams ND DM
LC3-II/β-actin
LC3-II/LC3I
D
Cyto-ID
pcDNA3
caPKCα
Puncta/cell
E
Cyto-ID
DMSO
SAG
Puncta/cell
F
Control siRNA+ 5 mM glucose
PKCα siRNA+ 5 mM glucose
Control siRNA+ 25 mM glucose
PKCα siRNA+ 25 mM glucose
Puncta/cell
Glucose (mM) 5 5 25 25
siRNA control + - + -
PKCα siRNA - + - +

Fig. 4A – 4F

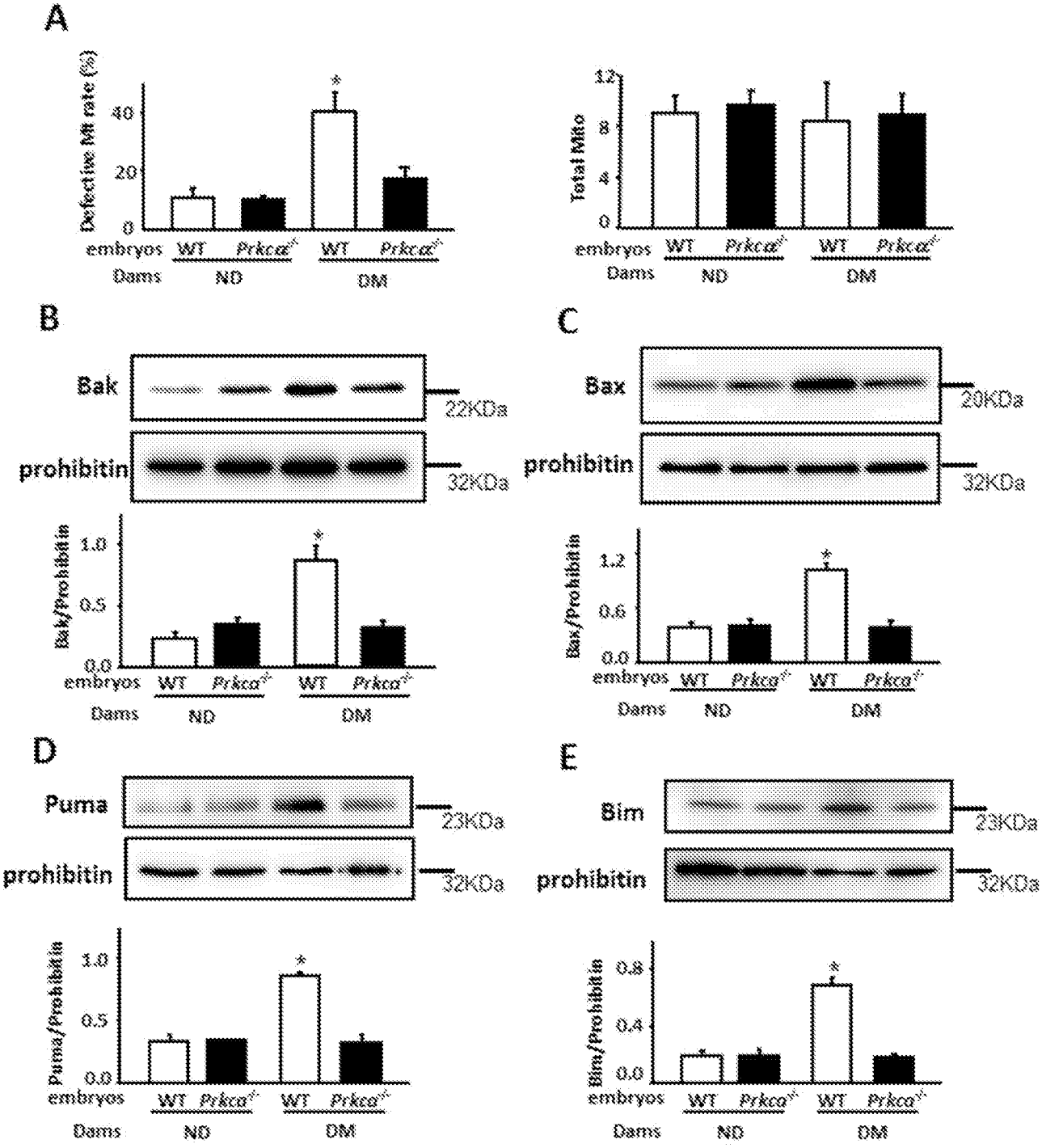

A
Defective Mt rate (%)
40
20
0
*
embryos WT Prkcα-/- WT Prkcα-/-
Dams ND DM
Total Mito
12
8
4
0
embryos WT Prkcα-/- WT Prkcα-/-
Dams ND DM
B
Bak
22KDa
prohibitin
32KDa
Bak/Prohibitin
1.0
0.5
0.0
*
embryos WT Prkcα-/- WT Prkcα-/-
Dams ND DM
C
Bax
20KDa
prohibitin
32KDa
Bax/Prohibitin
1.2
0.6
0.0
*
embryos WT Prkcα-/- WT Prkcα-/-
Dams ND DM
D
Puma
23KDa
prohibitin
32KDa
Puma/Prohibitin
1.0
0.5
0.0
*
embryos WT Prkcα-/- WT Prkcα-/-
Dams ND DM
E
Bim
23KDa
prohibitin
32KDa
Bim/Prohibitin
0.8
0.4
0.0
*
embryos WT Prkcα-/- WT Prkcα-/-
Dams ND DM

Fig. 5A – 5E

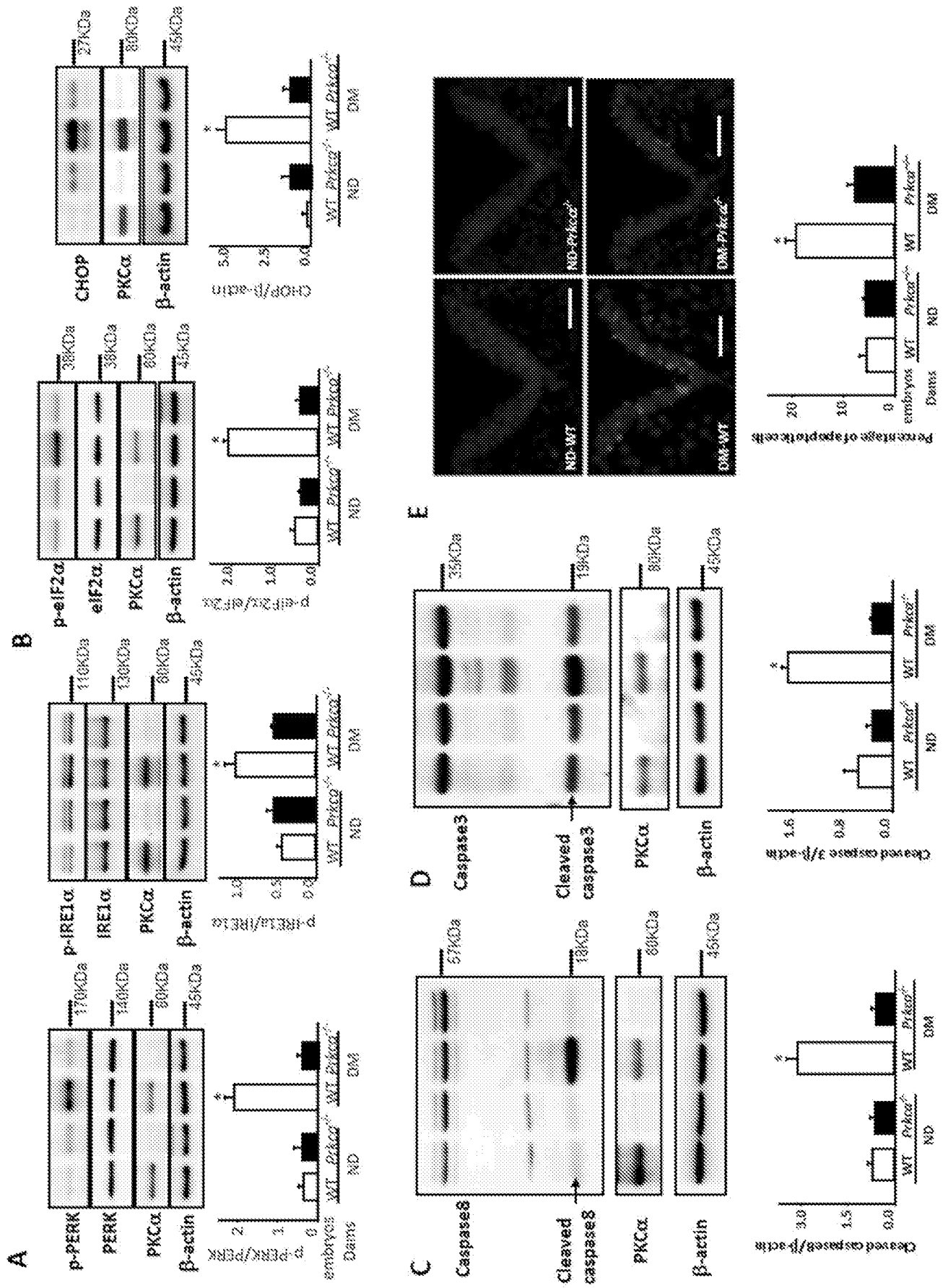

A
p-PERK
PERK
PKCα
β-actin
170KDa
140KDa
80KDa
45KDa
p-PERK/PERK
embryos
Dams
WT
Prkcα-/-
ND
DM
B
p-IRE1α
IRE1α
PKCα
β-actin
110KDa
130KDa
80KDa
45KDa
p-IRE1α/IRE1α
p-eIF2α
eIF2α
PKCα
β-actin
38KDa
38KDa
80KDa
45KDa
p-eIF2α/eIF2α
CHOP
PKCα
β-actin
27KDa
80KDa
45KDa
CHOP/β-actin
C
Caspase8
Cleaved caspase8
PKCα
β-actin
57KDa
18KDa
80KDa
45KDa
Cleaved caspase8/β-actin
D
Caspase3
Cleaved caspase3
PKCα
β-actin
35KDa
19KDa
80KDa
45KDa
Cleaved caspase 3/β-actin
E
ND-WT
ND-Prkcα-/-
DM-WT
DM-Prkcα-/-
Percentage of apoptotic cells

Fig. 6A – 6E

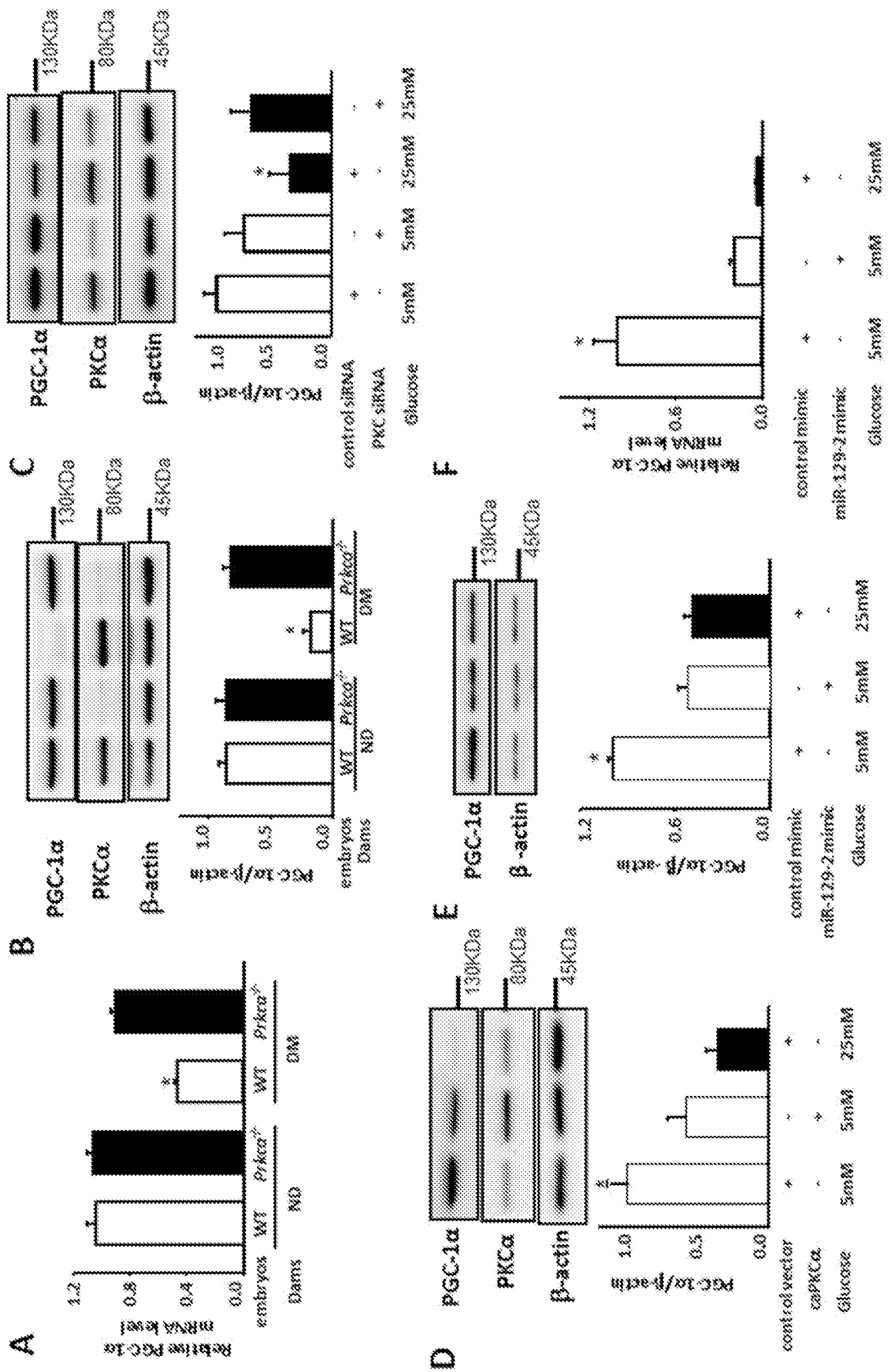

A
Relative PGC-1α mRNA level
1.2
0.8
0.4
0.0
embryos WT Prkca-/- WT Prkca-/-
Dams ND 0M
B
PGC-1α
PKCα
β-actin
130KDa
80KDa
45KDa
PGC-1α/β-actin
1.0
0.5
0.0
embryos WT Prkca-/- WT Prkca-/-
Dams ND 0M
C
PGC-1α
PKCα
β-actin
130KDa
80KDa
45KDa
PGC-1α/β-actin
1.0
0.5
0.0
control siRNA + - + -
PKC siRNA - + - +
Glucose 5mM 5mM 25mM 25mM
D
PGC-1α
PKCα
β-actin
130KDa
80KDa
45KDa
PGC-1α/β-actin
1.0
0.5
0.0
control vector + - + -
caPKCα - + - +
Glucose 5mM 5mM 25mM 25mM
E
PGC-1α
β-actin
130KDa
45KDa
PGC-1α/β-actin
1.2
0.6
0.0
control mimic + - + -
miR-129-2 mimic - + - +
Glucose 5mM 5mM 25mM 25mM
F
Relative PGC-1α mRNA level
1.2
0.6
0.0
control mimic + - + -
miR-129-3 mimic - + - +
Glucose 5mM 5mM 25mM 25mM

Fig. 7A – 7F

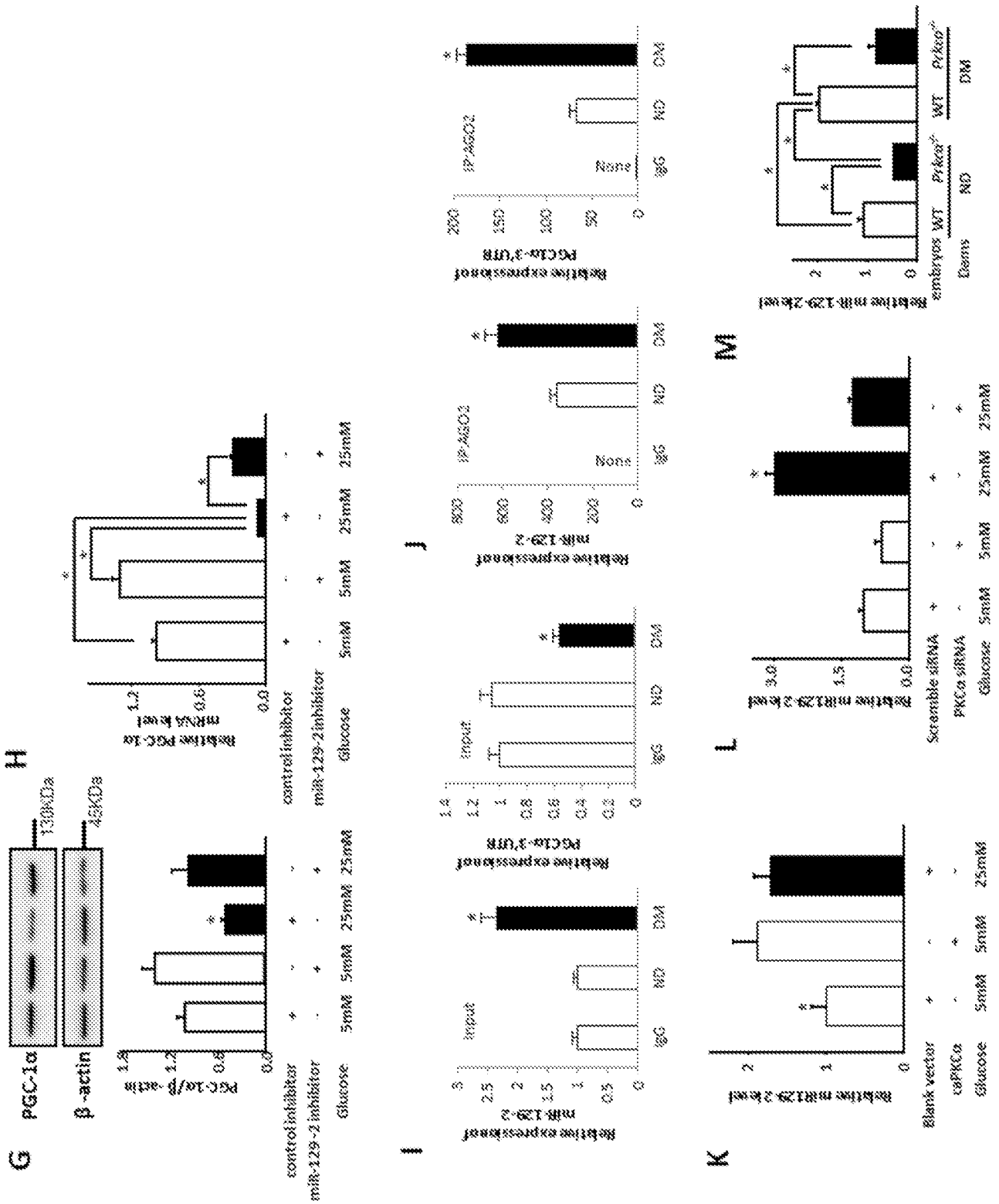

G
PGC-1α
130KDa
β-actin
45KDa
PGC-1α/β-actin
control inhibitor
miR-129-2 inhibitor
Glucose
5mM
25mM
H
Relative PGC-1α mRNA level
control inhibitor
miR-129-2inhibitor
Glucose
5mM
25mM
I
Input
IgG
ND
DM
Relative expression of PGC1α-3'UTR
Relative expression of miR-129-2
J
IP:AGO2
None
IgG
ND
DM
Relative expression of PGC1α-3'UTR
Relative expression of miR-129-2
K
Blank vector
caPKCα
Glucose
5mM
25mM
Relative miR129-2 level
L
Scramble siRNA
PKCα siRNA
Glucose
5mM
25mM
Relative miR129-2 level
M
Embryos
WT
Prkce-/-
Dams
ND
DM
Relative miR-129-2 level

Fig. 7G – 7M

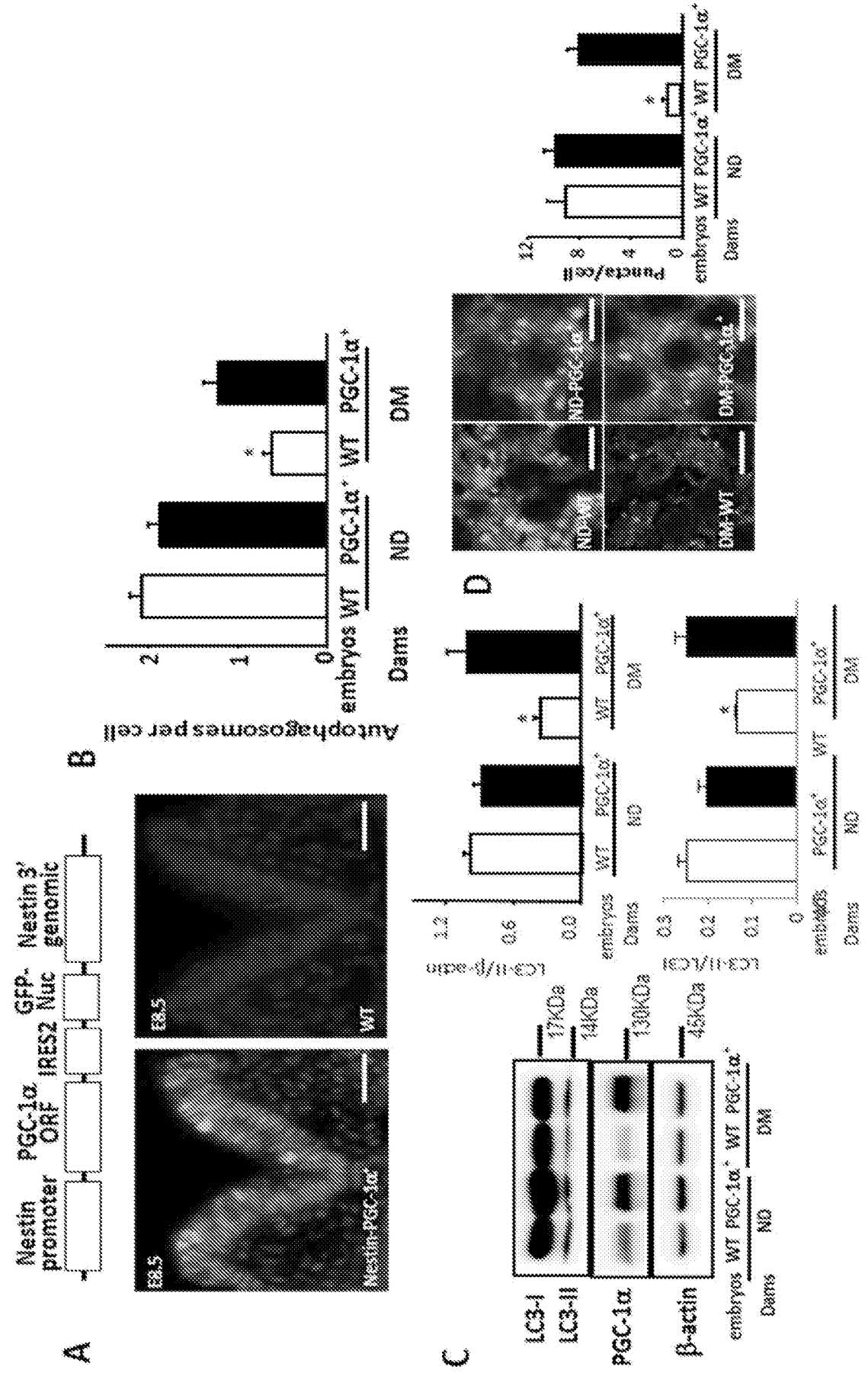

A
Nestin promoter
PGC-1α ORF
IRES2
GFP-Nuc
Nestin 3' genomic
Nestin-PGC-1α
WT
E8.5
B
Autophagosomes per cell
embryos WT PGC-1α WT PGC-1α
Dams ND DM
C
LC3-I
LC3-II
PGC-1α
β-actin
17KDa
14KDa
130KDa
45KDa
embryos WT PGC-1α WT PGC-1α
Dams ND DM
LC3-II/β-actin
LC3-II/LC3-I
D
ND-WT
ND-PGC-1α
DM-WT
DM-PGC-1α
Puncta/cell
embryos WT PGC-1α WT PGC-1α
Dams ND DM

Fig. 8A-8D

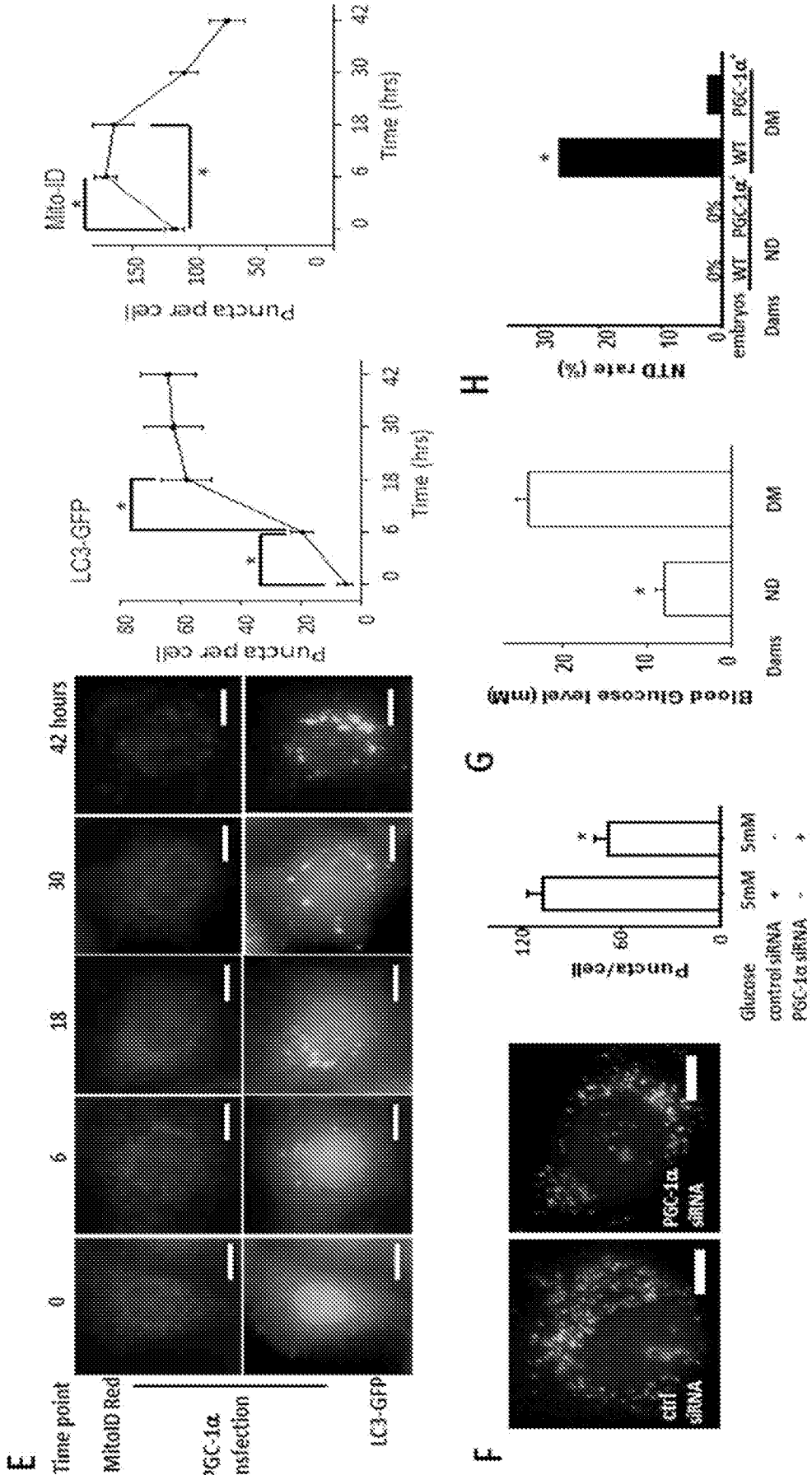

E
Time point
MitoID Red
PGC-1α transfection
LC3-GFP
0
6
18
30
42 hours
LC3-GFP
Puncta per cell
Time (hrs)
MitoID
Puncta per cell
Time (hrs)
F
ctrl siRNA
PGC-1α siRNA
Puncta/cell
Glucose
control siRNA
PGC-1α siRNA
5mM
5mM
G
Blood Glucose level (mM)
Dams
ND
DM
H
NTD rate (%)
embryos
WT
PGC-1α-
WT
PGC-1α'
Dams
ND
DM

Fig. 8E-8H

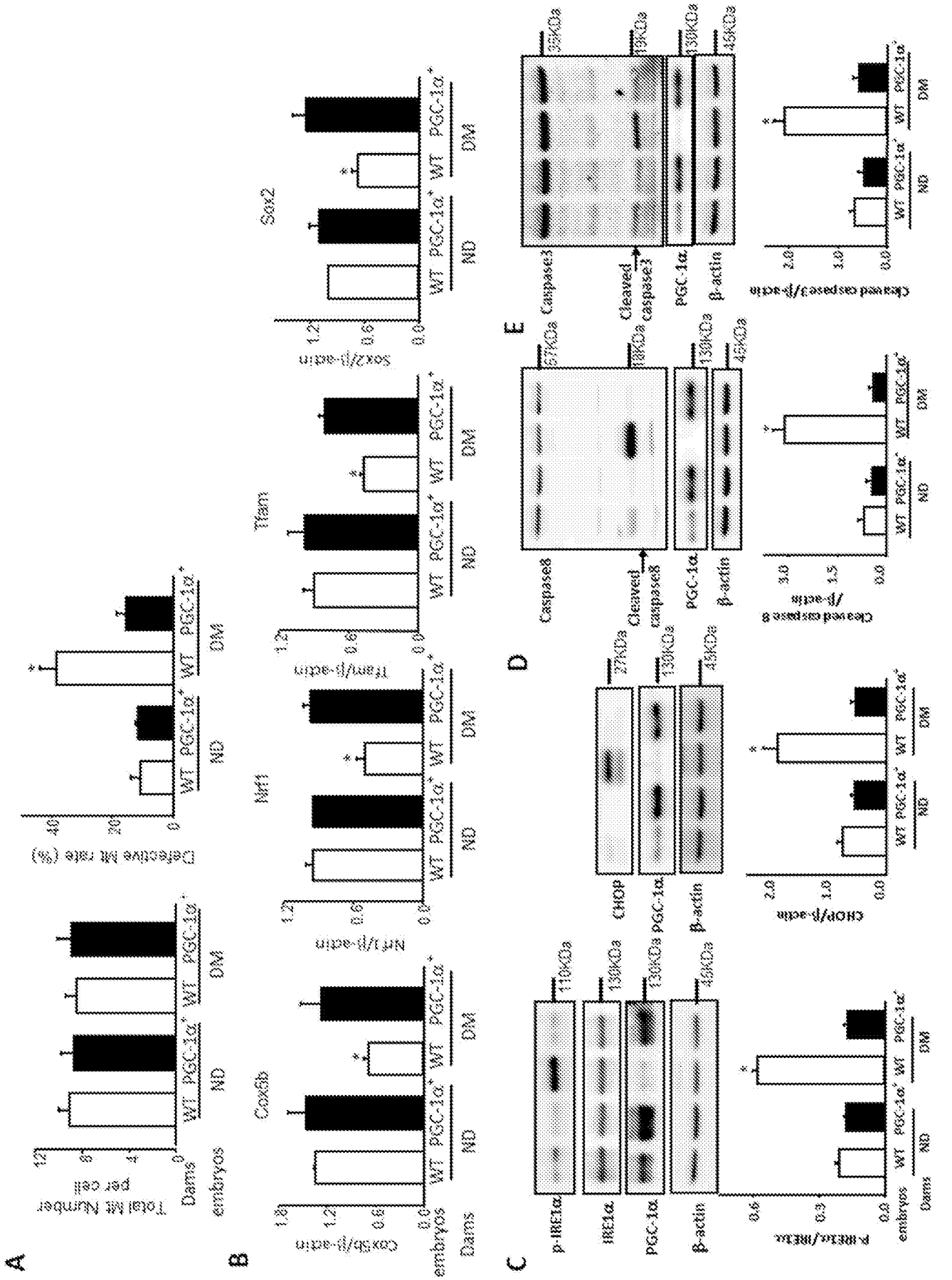
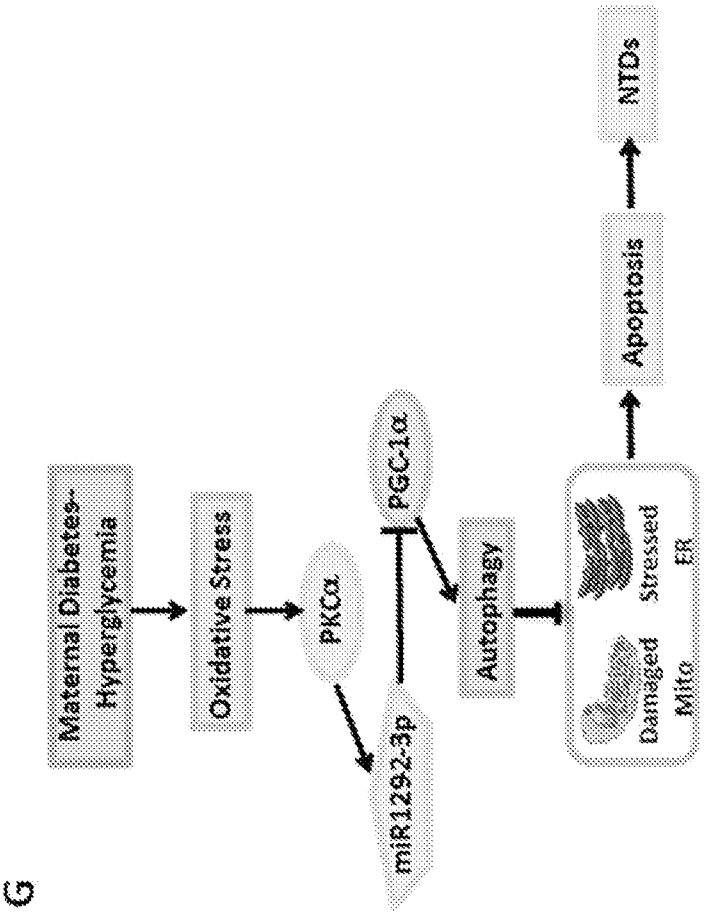

A
B
C
D
E
Total Mt Number per cell
Dams
embryos
ND
DM
WT
PGC-1α+
Defective Mt rate (%)
Cox5b/β-actin
Nrf1/β-actin
Tfam/β-actin
Sox2/β-actin
Cox5b
Nrf1
Tfam
Sox2
p-IRE1α
IRE1α
PGC-1α
β-actin
CHOP
Caspase8
Cleaved caspase8
Caspase3
Cleaved caspase3
110KDa
130KDa
45KDa
27KDa
18KDa
35KDa
19KDa
p-IRE1α/IRE1α
CHOP/β-actin
Cleaved caspase8 /β-actin
Cleaved caspase3/β-actin

Fig. 9A-9E


G
Maternal Diabetes-Hyperglycemia
Oxidative Stress
PKCα
miR1292-3p
PGC1α
Autophagy
Damaged Mito
Stressed ER
Apoptosis
NTDs

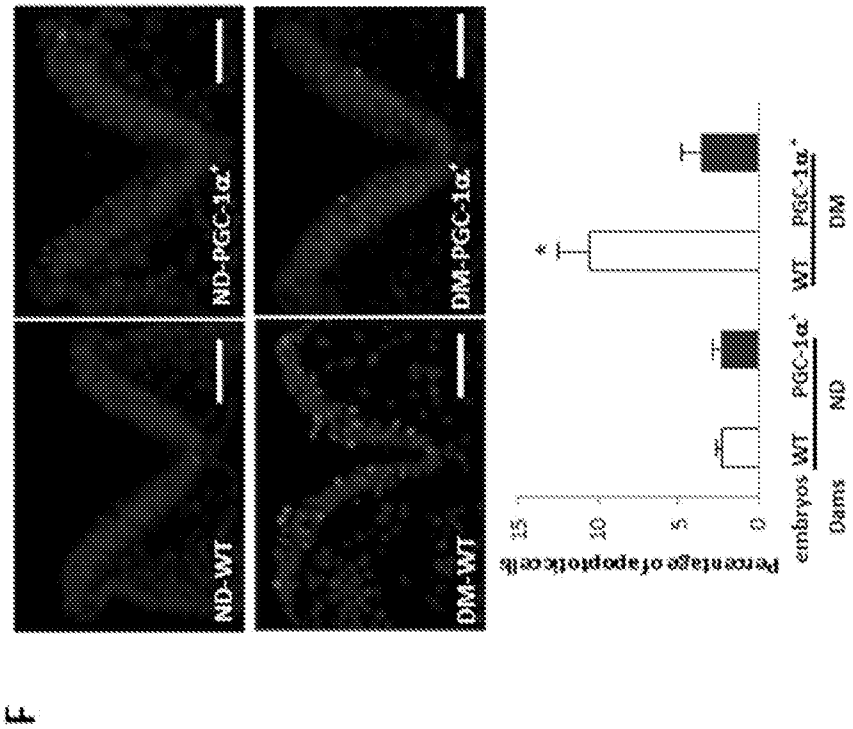
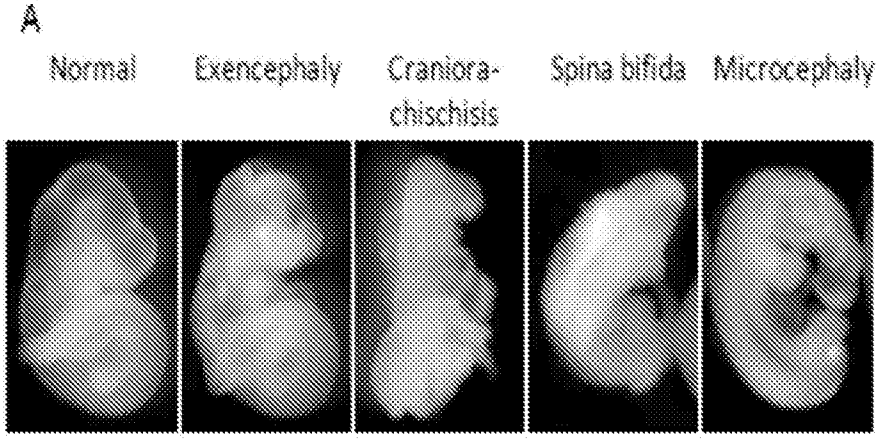

F
ND-WT
ND-PGC-1α+
DM-WT
DM-PGC-1α+
Percentage of apoptotic cells
15
10
5
0
*
embryos WT PGC-1α+ WT PGC-1α+
Dams ND DM

Fig. 9F-9G



NTD	Number of embryos	% among NTD Embryos
Exencephaly	52	74.3%
Craniorachisc-hisis	6	8.6%
Spina bifida	5	7.1%
Microcephaly	7	10.0%

36 litters; total 280 embryos.

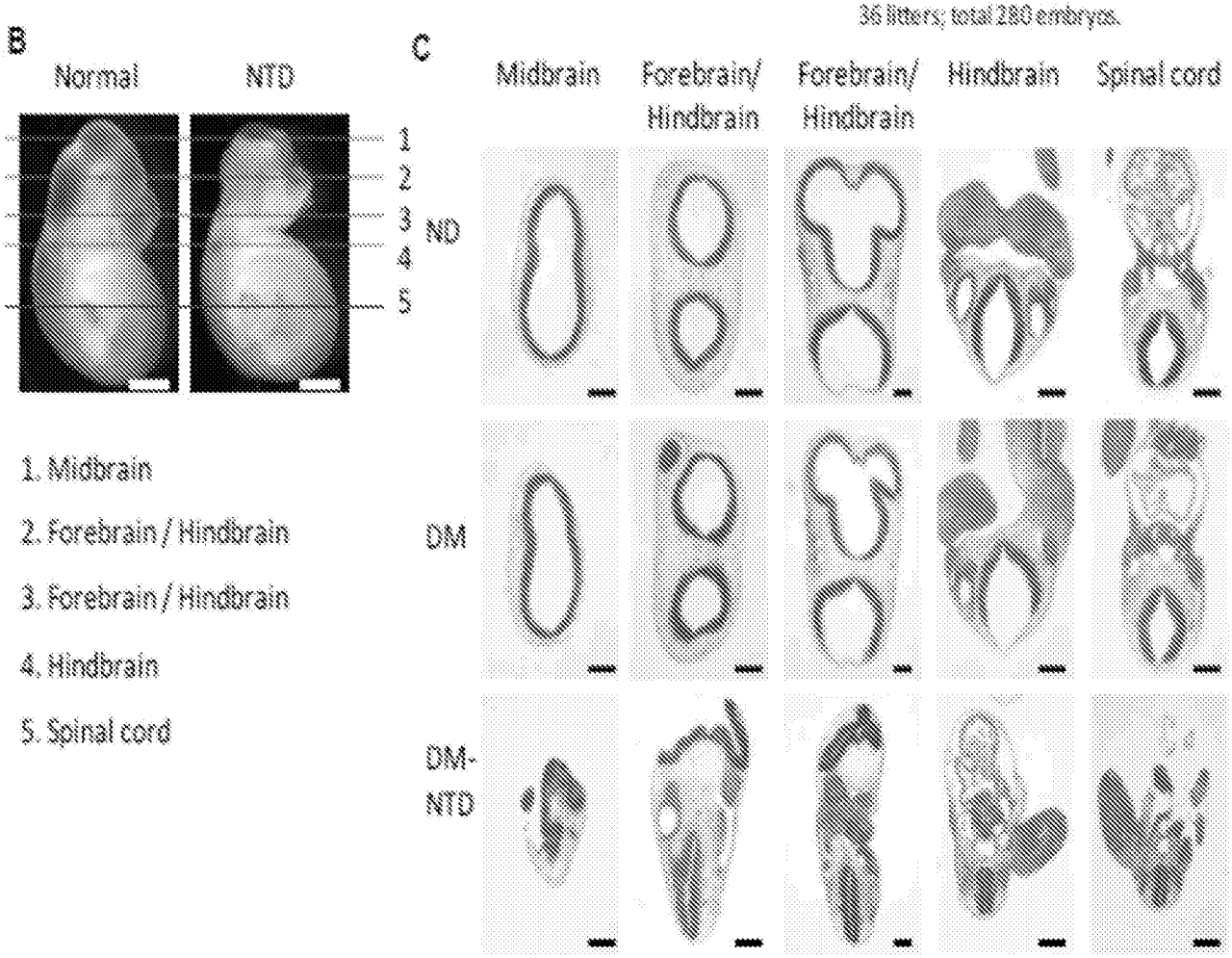


Fig. 10A-10C

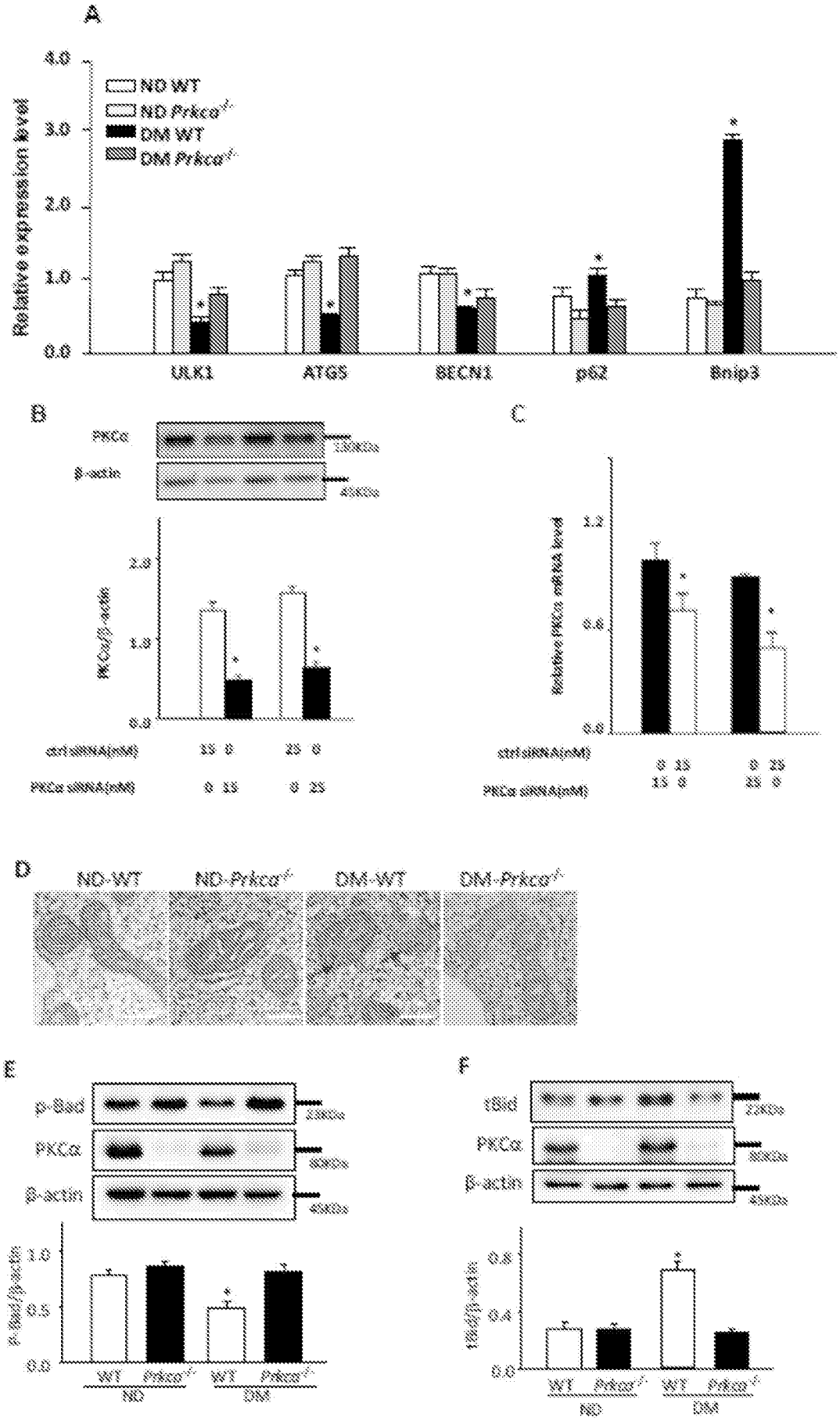

A
ND WT
ND Prkca-/-
DM WT
DM Prkca-/-
Relative expression level
4.0
3.0
2.0
1.0
0.0
ULK1
ATG5
BECN1
pG2
Bnip3
B
PKCα
β-actin
PKCα/β-actin
ctrl siRNA(nM)
PKCα siRNA(nM)
C
Relative PKCα mRNA level
D
ND-WT
ND-Prkca-/-
DM-WT
DM-Prkca-/-
E
p-Bad
PKCα
β-actin
WT
Prkca-/-
ND
DM
F
tBid
PKCα
β-actin

Fig. 11A-11F

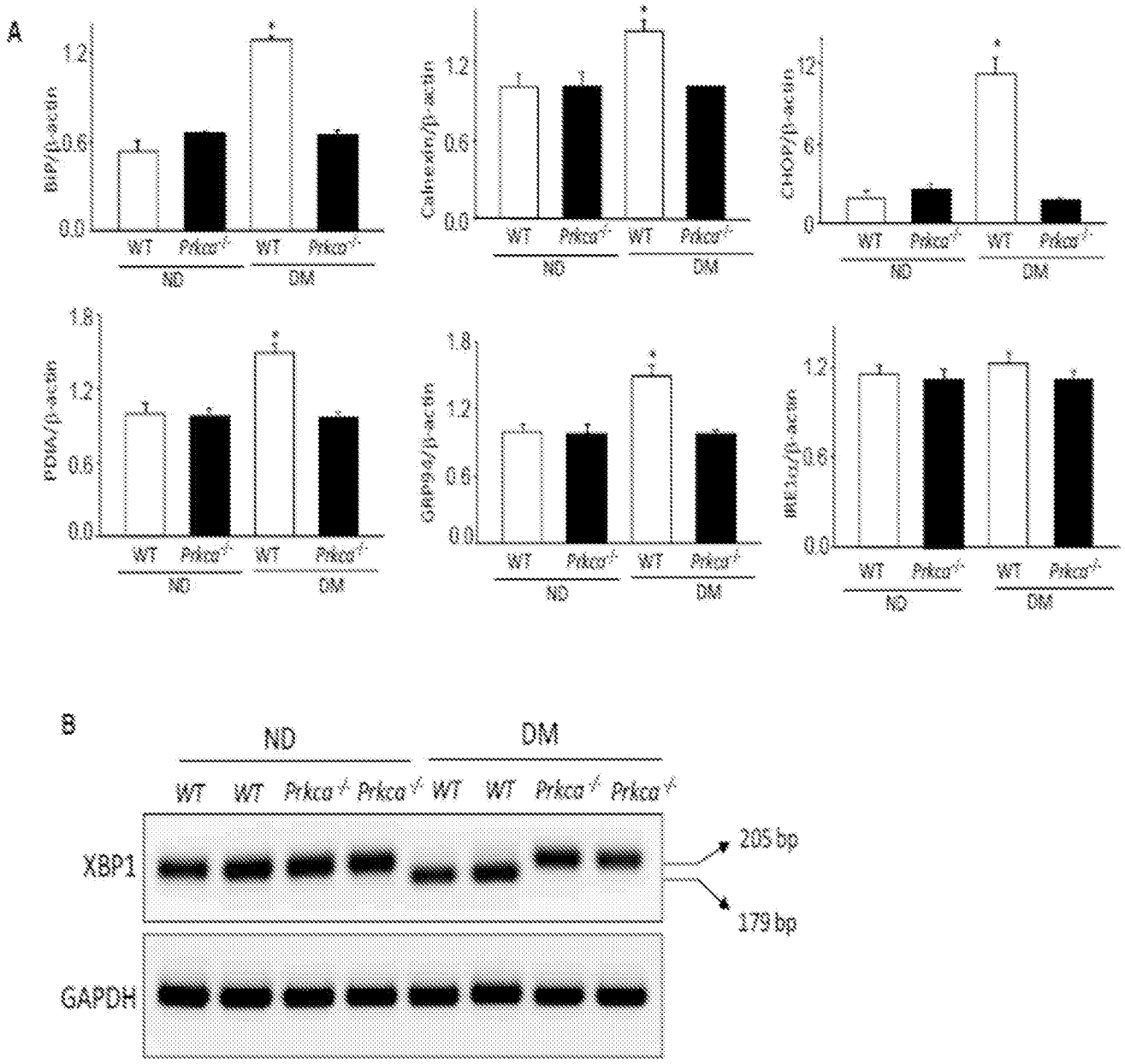

A
Bip/β-actin
Calnexin/β-actin
CHOP/β-actin
PDI/β-actin
GRP94/β-actin
Ire1α/β-actin
WT
Prkca-/-
ND
DM
B
ND
DM
WT WT Prkca-/- Prkca-/- WT WT Prkca-/- Prkca-/-
XBP1
205 bp
179 bp
GAPDH

Fig. 12A-12B

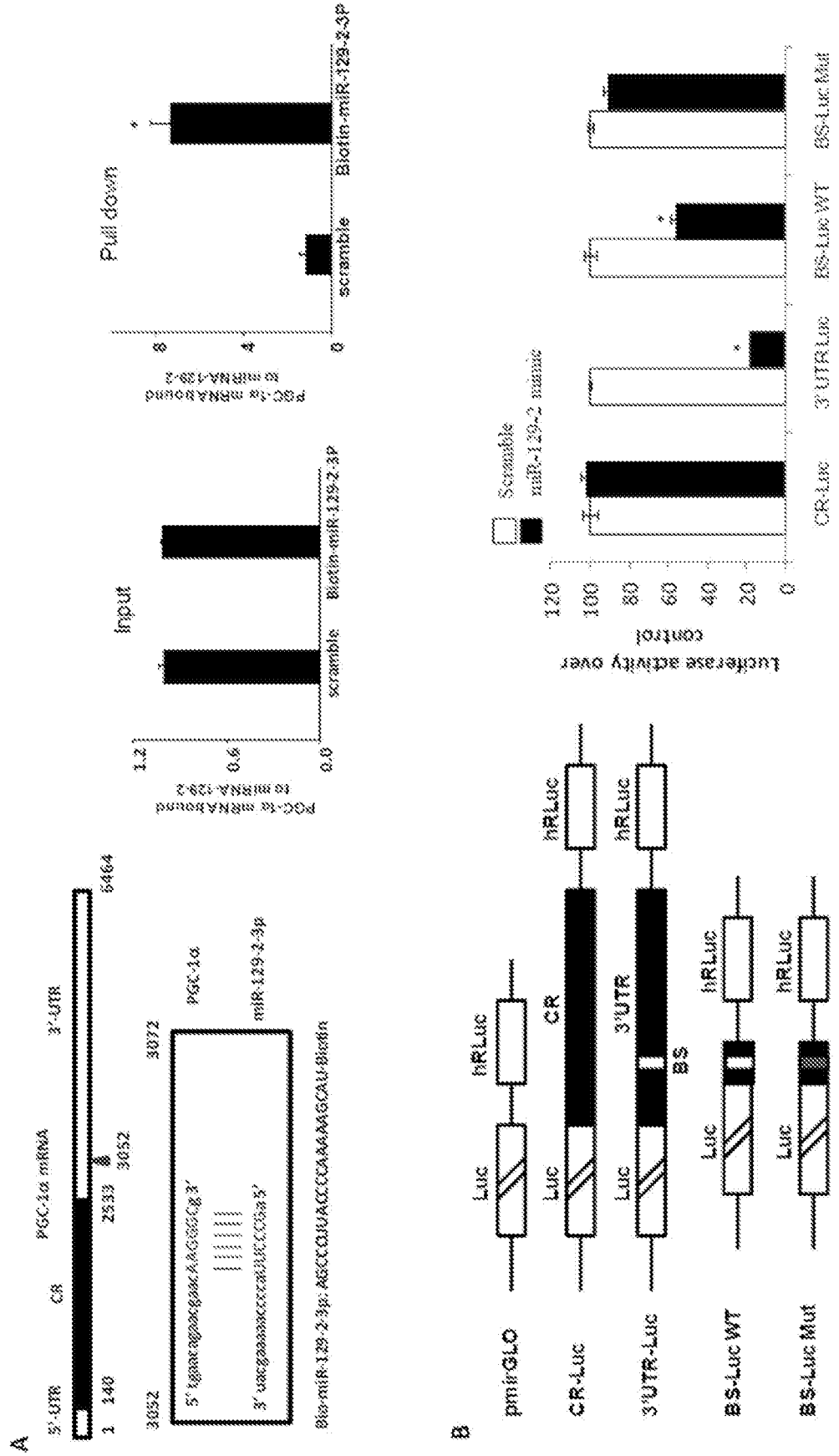

A
5'-UTR
CR
PGC-1α mRNA
3'-UTR
1
140
2333
3052
6464
3052
3072
PGC-1α
miR-129-2-3p
Bio-miR-129-2-3p: AGCCCUUACCCCAAAAAGCAU-Biotin
PGC-1α mRNA bound to miRNA-129-2
scramble
Biotin-miR-129-2-3P
Input
Pull down
B
pmirGLO
CR-Luc
3'UTR-Luc
BS-Luc WT
BS-Luc Mut
Luc
hRLuc
CR
3'UTR
BS
Scramble
miR-129-2 mimic
luciferase activity over control
CR-Luc
3'UTR Luc
BS-Luc WT
BS-Luc Mut

Fig. 13A-13B

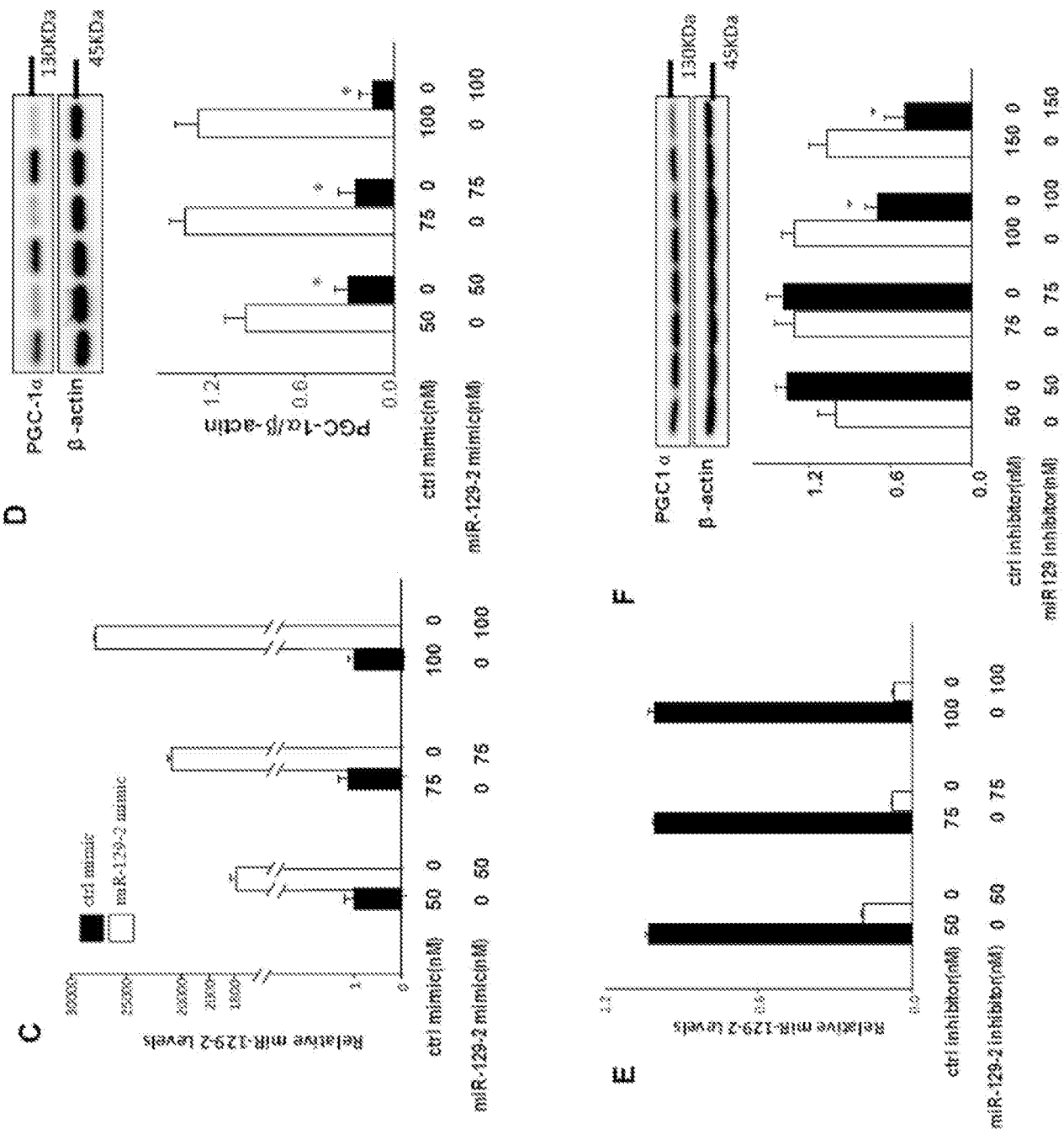

C
ctrl mimic
miR-129-2 mimic
Relative miR-129-2 levels
ctrl mimic(nM) 50 0 75 0 100 0
miR-129-2 mimic(nM) 0 50 0 75 0 100
D
PGC-1α
β -actin
130KDa
45KDa
PGC-1α/β-actin
ctrl mimic(nM) 50 0 75 0 100 0
miR-129-2 mimic(nM) 0 50 0 75 0 100
E
Relative miR-129-2 levels
ctrl inhibitor(nM) 50 0 75 0 100 0
miR-129-2 inhibitor(nM) 0 50 0 75 0 100
F
PGC1 α
β -actin
130KDa
45KDa
ctrl inhibitor(nM) 50 0 75 0 100 0 150 0
miR129 inhibitor(nM) 0 50 0 75 0 100 0 150

Fig. 13C-13F

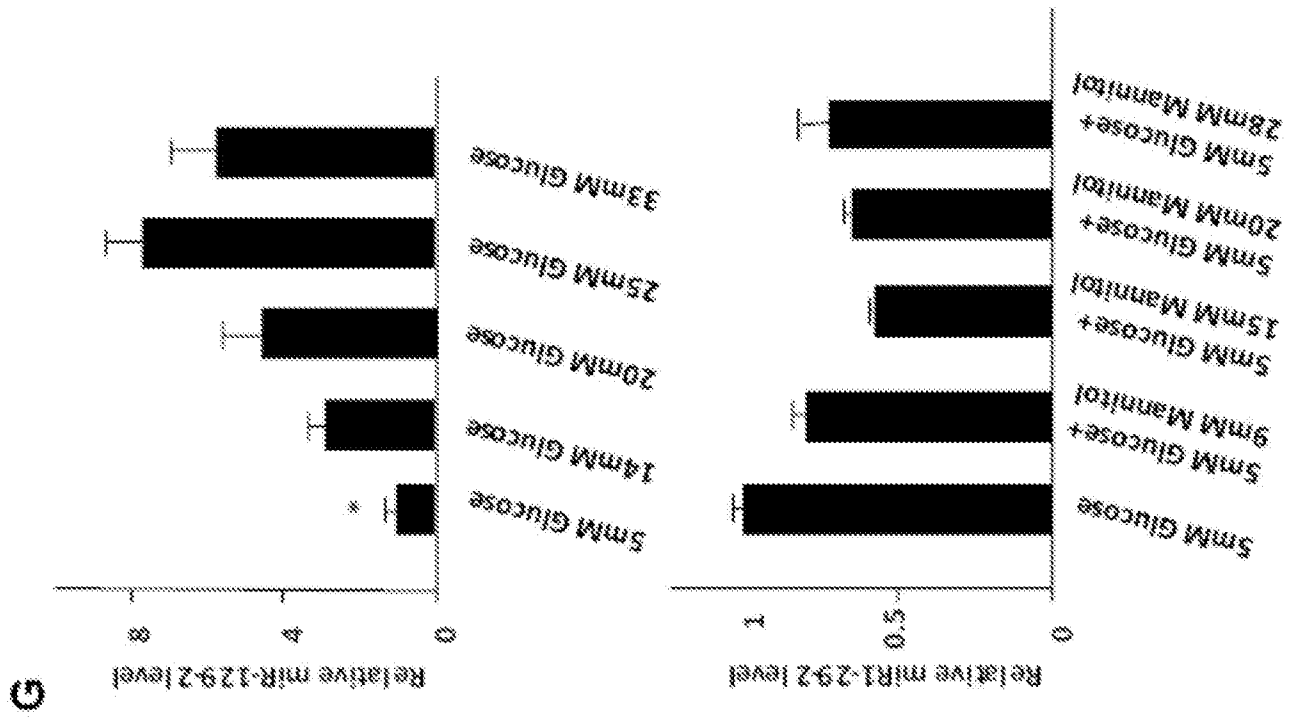

G
Relative miR-129-2 level
0
4
8
*
5mM Glucose
14mM Glucose
20mM Glucose
25mM Glucose
33mM Glucose
Relative miR1-29-2 level
0
0.5
1
5mM Glucose
5mM Glucose+ 9mM Mannitol
5mM Glucose+ 15mM Mannitol
5mM Glucose+ 20mM Mannitol
5mM Glucose+ 28mM Mannitol

Fig. 13G

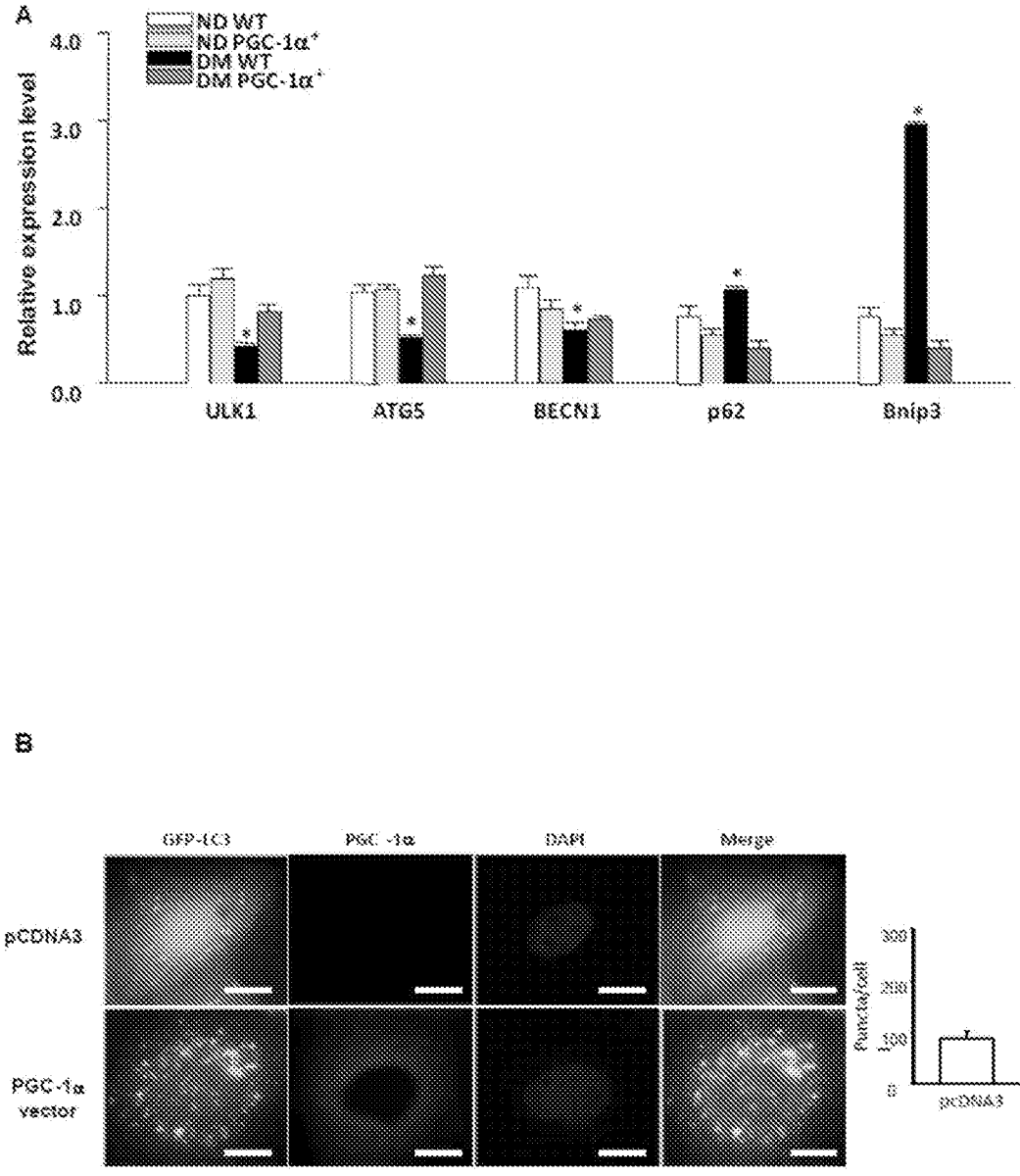

A
ND WT
ND PGC-1α+
DM WT
DM PGC-1α+
Relative expression level
4.0
3.0
2.0
1.0
0.0
ULK1
ATG5
BECN1
p62
Bnip3
B
GFP-LC3
PGC-1α
DAPI
Merge
pCDNA3
PGC-1α vector
Puncta/cell
300
200
100
0
pCDNA3
PGC-1α

Fig. 14A-14B

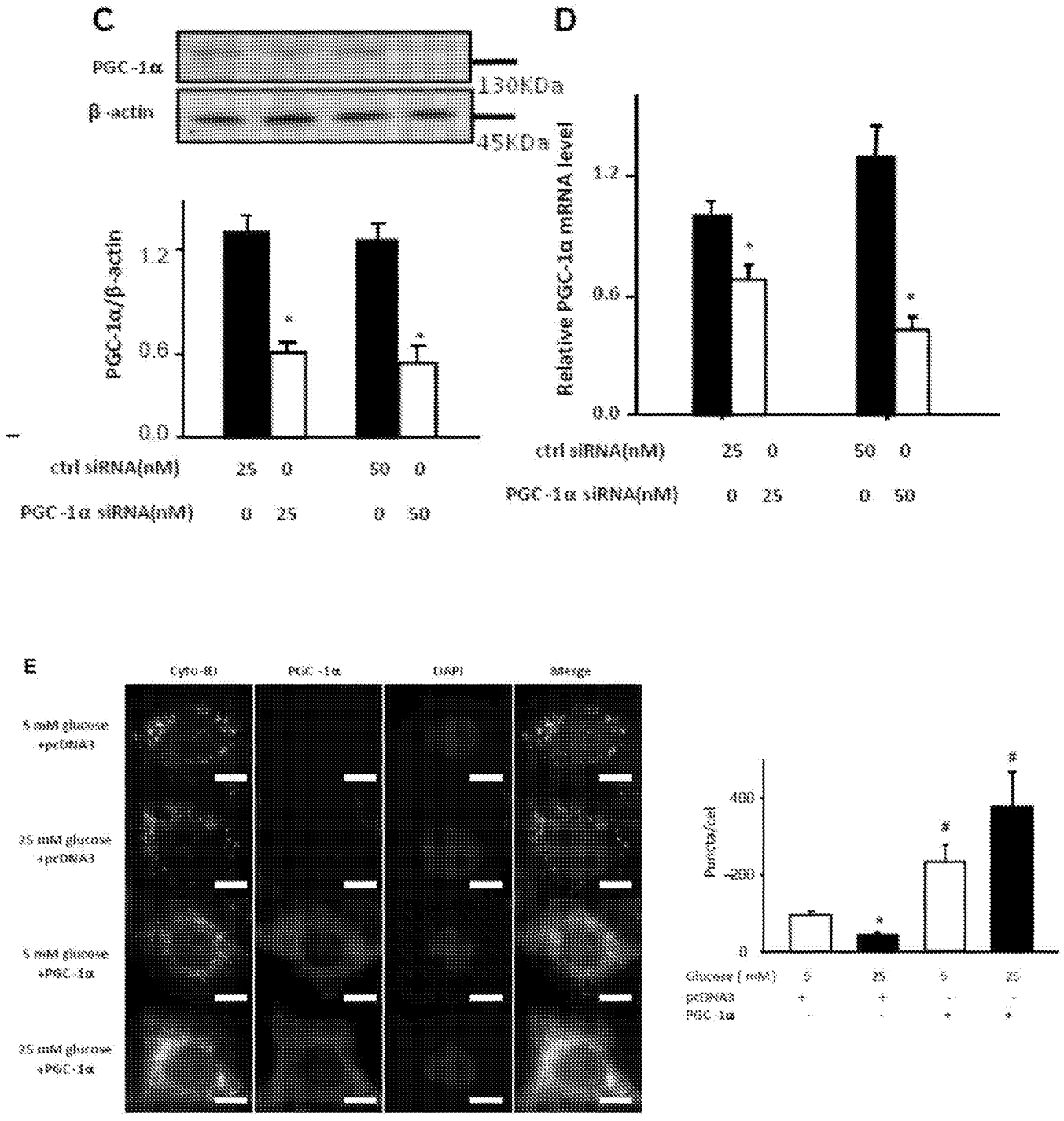

C
PGC-1α
β-actin
130KDa
45KDa
PGC-1α/β-actin
1.2
0.6
0.0
ctrl siRNA(nM) 25 0 50 0
PGC-1α siRNA(nM) 0 25 0 50
D
Relative PGC-1α mRNA level
1.2
0.6
0.0
ctrl siRNA(nM) 25 0 50 0
PGC-1α siRNA(nM) 0 25 0 50
E
Cyto-ID
PGC-1α
DAPI
Merge
5 mM glucose +pcDNA3
25 mM glucose +pcDNA3
5 mM glucose +PGC-1α
25 mM glucose +PGC-1α
Punctа/cel
400
200
0
Glucose (mM) 5 25 5 25
pcDNA3 + + - -
PGC-1α - - + +

Fig. 14C-14E

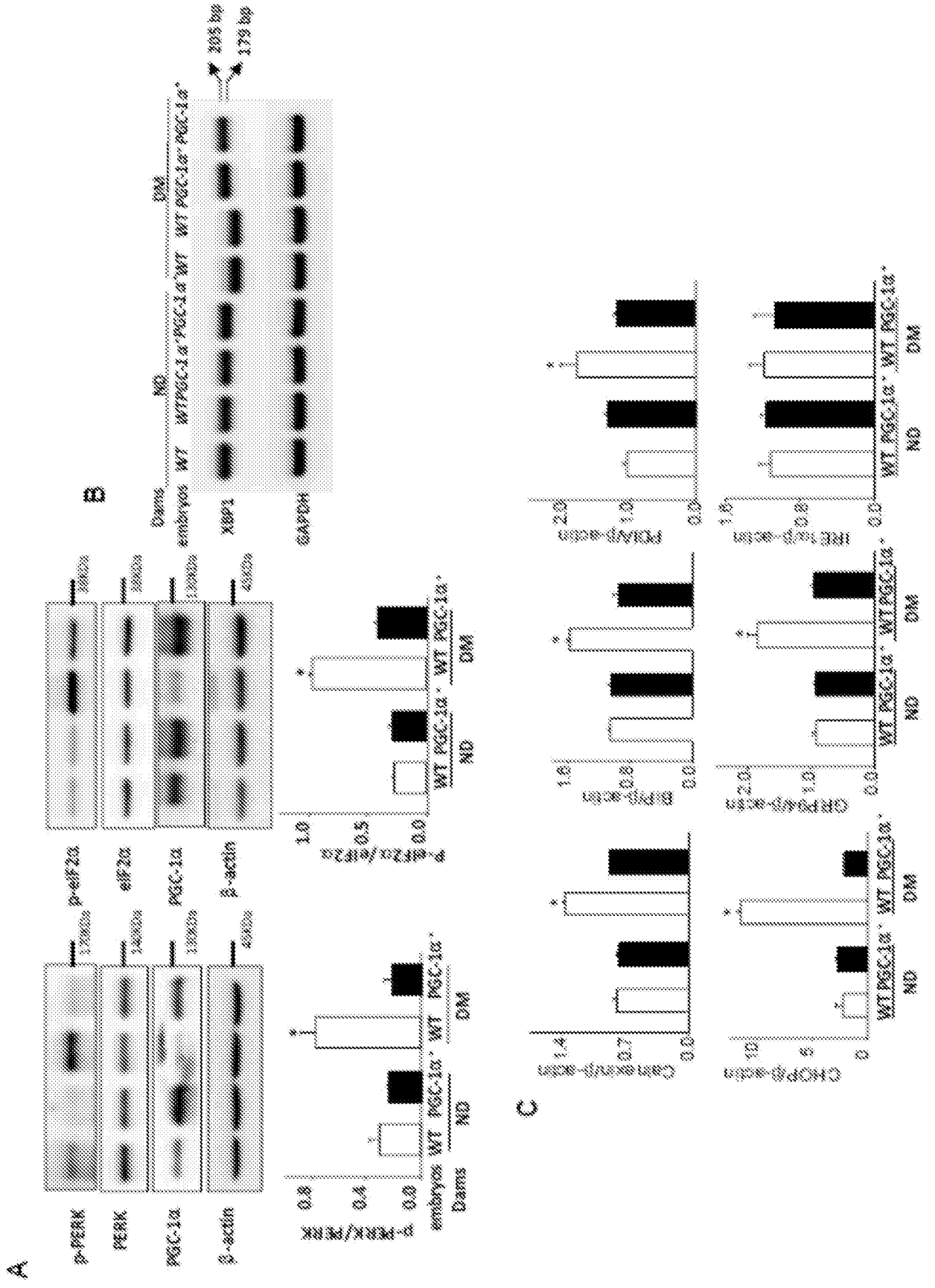

A
p-PERK
PERK
PGC-1α
β-actin
p-eIF2α
eIF2α
PGC-1α
β-actin
130kDa
130kDa
130kDa
40kDa
p-PERK/PERK
p-eIF2α/eIF2α
embryos WT PGC-1α⁻ WT PGC-1α⁻
Dams ND DM
B
Dams ND DM
embryos WT WT PGC-1α⁻ PGC-1α⁻ WT WT PGC-1α⁻ PGC-1α⁻
XBP1
GAPDH
205 bp
179 bp
C
Gen exon/β-actin
BiP/β-actin
CHOP/β-actin
FOXA2/β-actin
IRE1α/β-actin
WT PGC-1α⁻ WT PGC-1α⁻
ND DM

Fig. 15A-15C

	Experimental group	Glucose level (mM)	Total embryos	Total defect embryos	NTD rate (%)
ND	WT ♂ x WT ♀ (12 litters)	9.3±0.7	85	0	0
	Prkca$^{-/-}$ ♂ x *Prkca*$^{-/-}$ ♀ (11litters)	9.5±1.1	72	0	0
DM	WT ♂ x WT ♀ (12 litters)	26.1±1.1	78	23*	29.5*
	Prkca$^{-/-}$ ♂ x *Prkca*$^{-/-}$ ♀ (13 litters)	26.1±1.8	88	4	4.5

Fig. 16

Experimental group	Glucose level (mM)	Genotype	Total embryos	NTD embryos	NTD rate (%)
PGC-1α ♂ x ND-WT ♀ (13litters)	7.9±1.0	WT	47	0	0
		PGC-1α	45	0	0
PGC-1α ♂ x DM-WT ♀ (13 litters)	24.0±1.5	WT	44	10*	22.7*
		PGC-1α	46	1	2.2

Fig. 17

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2018/031159

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A61K 31/7106; A61K 31/74; A61K 39/395 (2018.01)
CPC - A61K 38/005; A61K 31/33; A61K 38/02 (2018.05)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC - 424/158.1; 424/145.1; 424/94.4; 424/130.1; 514/53 (keyword delimited)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2006/0069161 A1 (LEE et al) 30 March 2006 (30.03.2006) entire document	1-6, 13-15
X	WO 2011/066567 A1 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA et al) 03 June 2011 (03.06.2011) entire document	1, 7
X	US 2014/0235631 A1 (BUNT et al) 21 August 2014 (21.08.2014) entire document	1, 8-10, 17
X	US 2015/0299705 A1 (ASURAGEN, INC.) 22 October 2015 (22.10.2015) entire document	1, 11, 12
X	US 2013/0316971 A1 (YANG et al) 28 November 2013 (28.11.2013) entire document	1, 13-16
P, X	WANG et al. "Protein kinase C-alpha suppresses autophagy and induces neural tube defects via miR-129-2 in diabetic pregnancy," Nature Communications, 05 May 2017 (05.05.2017), Vol. 8, No. 15182, Pgs. 1-14. entire document	1-17
A	US 2010/0291069 A1 (REECE et al) 18 November 2010 (18.11.2010) entire document	1-17

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
27 June 2018	30 JUL 2018
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, VA 22313-1450 Facsimile No. 571-273-8300	Authorized officer Blaine R. Copenheaver PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774